



PHD

Life History Implications of Sex, Diet and Pathogen Exposure in the Fruit Fly

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Life History Implications of Sex, Diet and Pathogen Exposure in the Fruit Fly

Colin D. McClure

A thesis submitted for the degree of Doctor of Philosophy



University of Bath

Department of Biology & Biochemistry

November 2014

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List of Publications & Presentations

Publications

- McClure, C. D.**, W. Zhong, V. Hunt, F. Chapman, F. M. Hill & N. K., Priest. (2014). Hormesis results in trade-offs with immunity. *Evolution*. 68:2225-2233
- Zhong, W., **C. D. McClure**, C. R. Evans, D. T. Mlynski, E. Immonen, & M. G. Ritchie. (2013). Immune anticipation of mating in *Drosophila*: Turandot M promotes immunity against sexually transmitted fungal infections. *Proceedings of the Royal Society B: Biological Sciences*. 280:1-9
- Jensen, K., **C. D. McClure**, N. K. Priest & J. Hunt. (In review) Sex-specific effects of protein and carbohydrate intake on reproduction but not lifespan in *Drosophila melanogaster*. *Ageing Cell*
- Hunt¹, V. L., W. Zhong¹, **C. D. McClure**, D. T. Mlynski, E. M. L. Duxbury, A. K. Charnley & N. K. Priest (In review) Adaptive switch in life history strategy driven by cold-seeking behaviour in infected fruit flies. Submitted to *Proceedings of the Royal Society B: Biological Sciences*, ¹ Co-authors

Presentations - External to the University of Bath

- ‘Fungal-Infected Flies Self-Medicate through Diet’ - 47th annual ‘Population Genetics Group’ (PopGroup), University of Bath, 2014
- ‘Stress Genes Facilitate, and Immune Genes Suppress, Pathogenic Hormesis in the Fruit Fly’, 14th annual meeting of the European Society of Evolutionary Biology (ESEB), University of Lisbon, 2013
- ‘A Genetic Dissection of a Hormetic Response in *Drosophila melanogaster*’ - 46th annual PopGroup, University of Glasgow, 2013
- ‘Fungal Exposure Enhances Host Fitness in the fruit fly’ - 45th annual PopGroup, University of Nottingham, 2012
- ‘Pathogen’s Role in Ageing’ - 44th annual PopGroup, University of Hull, 2011
- ‘Microbes in Mortality’ - Bath Royal Literary and Scientific Institution public lecture, Bath, 2011 – Invited Speaker
- ‘A Fungal Treatment which Extends Life’ - 4th annual Scottish Ageing Conference, University of Glasgow, 2011
- ‘What Doesn’t Kill You, Makes You Fitter’ - Bath & Bristol Evolutionary Meeting, University of Bath, 2011 – **Poster**

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Thesis Summary

Understanding how organisms function is central to Biology. Assessing how animals respond to fluctuations in their environment and determining inter-individual variation in phenotypic plasticity is paramount to identifying the physiology of traits, the selective pressures which have shaped them, and how we can manipulate them to benefit human life. The over-arching goal of my thesis is to understand the effects of sex, diet and pathogen exposure on the physiology of the fruit fly to assess the versatility of their individual traits in response to these natural factors. Chapter 2 investigates how the sexes utilise nutrition towards their lifespan and reproduction, providing evidence that the reproduction of males and females requires different dietary components while lifespan does not. Chapter 3 reveals that the sexes also differ in how they utilise nutrients for pathogen resistance identifying that females are highly protein-limited and more susceptible to infection than males. Chapter 4 provides the first comprehensive study of how organisms alter their dietary intake in response to infection, finding that flies behaviourally ingest less and consume higher protein:carbohydrate ratio diets when exposed to live fungal spores. Chapter 5 explores the phenomenon of trait-enhancing external stresses, a response often termed hormesis. This study reveals that the beneficial physiological response from inactive fungal spore exposure, a potential form of hormesis, incurs immune costs. The implications of my results to the field of physiology are discussed in Chapter 6 where I also highlight the limitations of my work and potential consequences for life history research. Overall it is determined that studies investigating the natural physiological response of organisms or potentially beneficial treatments for our own species, must consider sex-specific effects, physiological consequences in a variety of traits, and how organisms may utilise variation within their environment to alter their phenotypic condition.

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General Introduction

1.1 Studying the Physiological Response

- How does diet affect an organism's ability to survive, reproduce and fight infection?
- How do these responses and an organism's dietary preference vary with sex?
- Does pathogen infection alter the nutritional demands and preferences of the host?
- Are the physiological benefits of dietary-restriction induced by calorie or nutrient changes and are they sex-specific?
- Do life-extending treatments incur 'hidden' physiological consequences?

Investigating the characteristics of organisms, how these differ between individuals and how they vary with environmental factors is central to Biology. Particularly in evolution and ecology, assessing within and between-organism variation is vital for our understanding of the selective pressures which have shaped their traits and therefore have profound implications for multiple disciplines, from immunology to ethology. However, with such wide inferences, it is important to ensure a common understanding of what physiological investigations are, the terms used, and their interpretations if the true impact of this research is to be appreciated.

1.1.1 Understanding Physiology

An organism's physiology is the way in which the organism functions, i.e. the mechanisms employed to ensure survival and reproduction. These measurable mechanisms or individual characteristics of an organism are known as traits (e.g. body mass, eye colour, etc.). The overall expression of the individual's traits in a given environment (e.g. 1.2µg, blue etc.), as well as its behaviour, is collectively known as the organism's phenotype.

All organisms differ in their phenotype which is a product of an individual's genotype (i.e. the nucleotide sequence and structure of an organism's genome), environment (i.e. temperature, diet etc.), genotype x environment interactions (i.e. environment-dependent gene-expression), and its interactions with other organisms (i.e. genotype⁽ⁿ⁾ interactions; e.g. pathogens, symbionts etc.; Bossdorf et al. 2008). All of an organism's traits arguably contribute to its Darwinian fitness (i.e. their ability to contribute genetic material to future generations) albeit to different extents. Traits can

therefore be likened to a hierarchy whereby particular traits of an organism, such as reproduction (i.e. the ability to acquire mates and produce offspring), growth and survival (i.e. the ability to avoid death) contribute substantially more to fitness relative to others (Agrawal et al. 2010). It can be realised that these 'higher' traits encompass an array of individual 'lower' traits which together, produce these measureable characteristics. Although it can be insightful to assess the expression of numerous individual traits, it is often only reasonable to assess a select few. As physiologists wish to investigate the evolutionary success of a given phenotype (i.e. phenotype-specific fitness values), researchers often assess 'higher' traits which are more 'visible' to eye of natural selection. As an organism's fitness can be crudely interpreted as the number of offspring produced throughout an organism's lifetime, the expression of reproduction (fecundity) and survival (lifespan) are often used as approximators of individual fitness (McGraw and Caswell 1996).

Reproduction is generally measured as the number of offspring an organism produces or of the effort and success in achieving matings within a given period. Survival is often simply measured as the number of days an organism lives however, with this information, it is difficult to disentangle the individual aspects of survival. Survival can consist of a variety of characteristics depending on the environment of the individual. In healthy cohorts, lifespan is influenced both by somatic cell maintenance and repair (i.e. rate of ageing) and the inherent likelihood of death (i.e. initial mortality; Bronikowski and Flatt 2010). In more stressful environments however, survival can predominantly be determined by pathogen or stress resistance as well as predator avoidance. Therefore to distinguish between these aspects of survival, it is important to assess the lifespan of individuals in environments where survival is limited by the particular characteristics of interest. Furthermore, with the development of mathematical modelling procedures using sufficiently large populations, the individual characteristics of mortality within healthy ageing cohorts can be separated with a degree of confidence (Pletcher 1999; Bronikowski and Flatt 2010). Therefore, through fairly simple experimental procedures, trait measurements and environmental manipulations, we can identify how an organism's physiology responds to particular environments, the fitness levels of these responses, and how these vary between individuals and populations.

1.1.2 The Importance of the Physiological Response

A major aim of Biology is to understand the variation displayed within nature and to apply this knowledge to better the quality of human life. Investigating the physiological response assists in this aim as it informs us of how individual traits of an organism respond to particular environmental fluctuations (termed reaction norms) and the extent at which they can be environmentally or genetically manipulated (Sarkar and Fuller 2003; Bossdorf et al. 2008).

Although trait expression can vary between environments (e.g. diet, temperature etc.), the extent of variation (i.e. plasticity) is limited to a set range. This limit is largely determined by the genetic component of a trait and predominantly defines its heritability, while its plasticity is a result of environmentally-induced variation (Sarkar and Fuller 2003). A primary example of this is height in human populations which has been found to be up to 90% heritable, leaving approximately 10% variability within the trait (Weedon et al. 2008). From knowledge of the genetic and non-genetic trait contributions, biologists can use environmental or genetic manipulation (e.g. through selection experiments) to maximise the expression of particular traits. Long standing examples of this can be seen in developing the yield of agricultural animals and plants through optimising diet/soil composition and continued artificial selection (Diamond 2002).

A unique system to investigate genetic variation between traits is that displayed between the sexes. Many organisms exhibit gender differentiation such that each sex experiences specific trait optima and plasticity due to the varying pressures of sexual selection (Fanson et al. 2013). Understanding why differences in sexual selection exist between the genders, and its consequences for the expression and evolution of traits, is important if we are to grasp the mechanistic components underlying these selected traits. Only with appreciating the extent of sex-specific variation can we progress to develop therapies or treatments to improve the quality of life of both genders.

Another important consideration for physiologists is also to investigate the consequences of trait improvement on the wider phenotype of the organism. An often observed phenomenon in ecological studies is that certain trait benefits (e.g. lifespan) deter the expression of another (e.g. fecundity; Flatt 2011). As many, if not all, traits contribute to the overall fitness of an organism, implications of a physiological response on other traits should be, but on occasion aren't, considered (Forbes 2000). Therefore,

although varying a condition may result in a beneficial expression in one trait, the same conditions may lead to a detrimental expression in another which may or may not affect the fitness of the organism (Zera and Harshman 2001). From these so-called physiological or phenotypic ‘trade-offs’, it is vital for biologists to assess a variety of traits of interest when assessing the reaction norms of a particular characteristic (Agrawal et al. 2010). This is paramount when determining particular conditions which may improve the quality of life in humans.

1.1.3 The Rise of Model Organisms

Assessing physiological responses in highly complex organisms such as ourselves, can often lead to inconclusive results due to the vast array of factors (both genetic and environmental) which may influence the data. Therefore using simpler organisms of which we share similar characteristics but who have less sophisticated interactions with their environment are used to gain insights into these questions.

Brewer’s yeast, *Saccharomyces cerevisiae*, is a uni-cellular eukaryote which has often been used to identify physiological responses, such as stress resistance, and cellular processes, through genetic manipulation (Wullschleger et al. 2008). Although this species has been invaluable in our understanding of the cell cycle and the processing of nucleic acids, yeast lacks the complexities and interactions experienced by multi-cellular organisms. *Caenorhabditis elegans*, a free-living nematode, is the most fundamental multi-cellular model organism used for biological investigation. With a mass of genetic information and tools available, studies of *C. elegans* have been substantially insightful in determining the genetic basis of ageing, amongst other traits (Murphy et al. 2003). Despite the versatility of this organism, their reproduction is primarily asexual which, although useful in creating isogenic lines, provides challenges to investigate variation between the sexes (Byerly et al. 1976).

The mouse, *Mus musculus*, has two genders, reproduces exclusively through sex, and shares a large degree of genetic similarity (i.e. share over 90% gene equivalents) with humans (Waterston et al. 2002). However, with all mammals, mice are endothermic and thus maintain a complex homeostatic internal environment making it difficult to identify particular factors which elicit individual physiological or genetic effects (Kemp 2006). Additionally, mice have a long generation time and are difficult to culture relative to

invertebrate model species. This limits the statistical power at which physiological or genetic effects can be identified due to the low level of replication which can be achieved in experimental design. Clearly a happy medium needed to be identified.

Drosophila melanogaster, the common fruit fly, has been employed in laboratory investigations for approximately a century (Beckingham 2007). These organisms are easy to culture, have short developmental and generation times, can live in relatively high density populations, exist as two genders and reproduce sexually providing an opportunity to assess sex-specific differences. Furthermore, fruit flies, as ectotherms, are amenable to laboratory manipulation as their internal physiology can be altered simply by adjusting the ambient temperature (Heerwaarden et al. 2012). Consequently, with the progress of genomics, *D. melanogaster* has been one of the first species to have its genome sequenced and annotated at the turn of the millennium (Ashburner and Bergman 2005).

As flies share just over 70% of their genetics with humans (Rubin et al. 2000), investigating the genetic underpinnings of their traits and changes in expression levels due to various environmental interactions can be insightful for our own physiology. Along with the sequencing and annotation of the *Drosophila* genome came rapid development in gene silencing capabilities. The RNA interference (RNAi) system for *D. melanogaster* has almost limitless potential (Dietzl et al. 2007). Researchers cannot only suppress any of the vast majority of genes within the *Drosophilid* genome, but can also target knock-downs in selected organs with the use of tissue-specific drivers. This advance has been a significant revelation, securing *Drosophila* as one of the most resourceful model species in biology today (Kohler 1994; Beckham 2007). Therefore investigations of *Drosophila* can inform us of the differences and evolution of the sexes, as well as assisting our understanding of the molecular underpinnings and plasticity of our own physiological traits.

1.2 Genetic Variation in Physiology

As acknowledged previously, the differences in traits observed between organisms are substantially determined by the genetics of the organism. Larger organisms are generally known to live longer and have a much lower rate of reproduction than those of a much smaller size as a result of selection pressures enforced from their environments (Stearns 1976). Understanding the basis of these genetic differences is imperative to determining the molecular mechanisms which underlie traits such as longevity so that we can manipulate them. Studies using the molecular tools of model species, such as *C. elegans* and *D. melanogaster*, have uncovered genes which underlie these traits. Mutations in, or the repression of, specific molecules such as insulin growth factor receptor Daf-2, the transcription factor DAF-16 (FOXO) or the protein ribosomal S6 Kinase (S6K) can extend lifespan by up to 454% when in combination (Chen et al. 2013). Despite the effective use of molecular genetic systems in model systems, much is still unknown over the genetic regulation of survival and reproduction between individuals and species.

A useful system in nature to assess the underlying genetics of particular traits is to investigate the physiological differences displayed between the sexes. Although the sexes share the vast majority of their genetic material, sex-specific gene expression accounts for much of the differences observed (McIntyre et al. 2006). Understanding how the sexes vary in their individual traits may shed light on the effects of sexual selection as well as the genetic and environmental contributions of these characteristics.

1.2.1 Variation Between the Sexes

Male and female animals have been shown to differ considerably in lifespan (somatic cell maintenance), pathogen resistance (immune activity and susceptibility), reproduction (e.g. gamete production & maintenance and parental care) and their response to environmental stimuli (Holtby and Healey 1990; Bonduriansky et al. 2008). To understand the sex-specific selection which has resulted in these trait differences, we must first identify the underlying selective pressures which produced anisogamy (divergent gametic size; i.e. the universal characteristic of sex).

Following the formation of two mating types, anisogamy is considered to have arisen from the dysregulation of isogamy (equal gametic size) in the model presented by Parker et al. (1972). In this 'gametic size' model it is theorised that multicellular organisms

which reproduce through the fusion of isogamous gametes will exhibit a level of genetic variation between the size and/or number of gametes produced. The model suggests that selection will favour the divergence of gamete size (i.e. anisogamy) such that small gametes (sperm) will parasitise the investment of the larger gametes (egg) to the zygote, and that selection imposed from the sperm will maintain the disassortative mating between mating types (Parker et al. 1972). Although this model is widely accepted to reconstruct the properties of the sexes, some of the assumptions on which the model is based, namely that zygote fitness is, at least at times, disproportionate to its size, have received inconclusive or unsupportive evidence in model organisms (i.e. *Volvocales*; Randerson and Hurst 2001). More recent work using discrete cell divisions as optimising trait values instead of gamete size, can account for the variation in gamete pairing in the *Volvocales* and fungi from random mutations and the specific ecological conditions (i.e. gametic encounter rates and selection pressure on zygote survival) of the populations (Togashi et al. 2012).

Despite the uncertainty of how the variation between gametes arose, once the sexes had formed, intra-sexual selective pressures drove the variation in physiology that we observe between the sexes today. As a result of anisogamy, the sex producing small motile gametes (i.e. males) experience substantial fluctuations in their Darwinian fitness than those which produce large, often immotile, gametes (i.e. females; Bateman 1948). This is because sperm must compete with each other to fertilise eggs. From this basic theory, we can infer that males are often fitness-limited through their access to eggs, a scenario which encourages competition between males. Females, on the other hand, generally do not have to compete for matings, and thus fitness-limited by their egg production rate (Trivers 1972). In these scenarios, males benefit from prioritising reproductive effort and competitive ability while females invest primarily in direct germ cell investment and longevity (Clutton-Brock and Isvaran 2007; Bonduriansky et al. 2008). These variations in optimal sex-specific phenotypes are referred to as the Bateman's principle and underlie sexual selection (manifested through male-male competition and female choice of mating partner; Bateman 1948; Harvey and Godfray 2001; Rolff 2002).

As suspected by evolutionary theory, the sexes have been found to differ in these respects. In many populations, females are found to have reduced rates of ageing than males and higher immune investment (Fanson et al. 2013; Regan and Partridge 2013).

However, more data is coming to light to suggest that the differences between males and females are not always so clear (Lindsey and Altizer 2008). Furthermore, recent investigations have identified sex-specific evolutionary rates in lifespan highlighting the possibility that each sex can develop independent mechanisms of ageing (Lehtovaara et al. 2013). It is clear that further studies into the differences between the sexes both in terms of their traits and phenotypic plasticity are required if we are to gain insights about the underlying genetics of these characteristics and the selective pressures driving them.

1.3 Environmental Variation in Physiology

In addition to having a genetic component, a significant proportion of an organism's phenotype depends on its environment (McNamara and Houston 1996). Researchers have used a number of techniques to assess the physiological response to both biotic and abiotic factors and how organisms can utilise environmental variation to benefit their individual traits. Commonly, the influence of temperature has been used particularly in ectotherms as their metabolic rates are determined by ambient temperature (Gillooly et al. 2001; Kenyon 2010). Frequent, intimate interactions between organisms and their environment are through diet and the interaction with potential pathogens and parasites (Cotter et al. 2011). It is important to assess how these environmental interactions affect trait performance if we are to understand the true physiology of the organism in its natural conditions and the context in which their traits have evolved.

1.3.1 Diet and Nutrition

There are two main aspects to consider of an organism's diet. First, the overall number of calories ingested (i.e. quantity) can have drastic effects on an organism's physiology. This was identified almost 80 years ago with McCay et al.'s ground-breaking experiment which found that restricting the diet of rats led to lifespan extension (McCay et al. 1935). This finding quickly led to the phenomenon of calorie restriction which has been considered a treatment for extending life in humans (Fontana et al. 2010). The second important aspect of diet is its composition. The various traits of an organism demand the use of specific nutrients (Lee et al. 2008). It is therefore paramount to be able to determine which components of diet are required for individual traits and how organisms utilise their diet to express their phenotype, however these have proved difficult tasks.

Experimental dietary manipulation has been used for over a century (Osborne and Mendel 1913). The majority of early experiments utilising dietary manipulation have emerged from two primary themes of research; one attempting to further understand the nutritional effects of dietary restriction found in rodents (McCay et al. 1935; Ross 1961; Hsueh et al. 1966; Fernandes et al. 1976; Weindruch et al. 1986), and the other assessing growth rates, milk-yields, and disease resistance of livestock (Lecce et al. 1961; Storry and Rook 1964; Dobson and Bawden 1974). The advantages of the invertebrate system were soon realised and nutritional studies investigating diet-induced longevity extensions and

physiological shifts were undertaken in insects, namely *Drosophila* (Chippindale et al. 1993; Simmons and Bradley 1997). Adopting insects as a model system rapidly accelerated the investigation of diet in physiology identifying the importance of protein in egg production and immune function and carbohydrates in longevity (Vass and Nappi 1998; Bauerfeind and Fischer 2005; Lee et al. 2006). Although these studies provided novel insights into the nutritional requirements of individual traits, the methods for dietary manipulation resulted in the inability to separate the effects of energy and specific nutrient consumption.

In the studies mentioned above, dietary or calorie restrictions are commonly implemented through limiting the amount of food available or diluting the resource. Likewise altering the levels of carbohydrate and protein (two prominent macronutrients required by animals; see Lee et al. 2008) involved directly increasing the proportion of macronutrient source in the diet, often resulting in the dilution of other dietary components (salts, vitamins, lipids etc.; Tatar 2011). As micronutrients are known to influence lifespan and reproduction, any effects attributed to protein or carbohydrate availability/restriction from this method of dietary manipulation may be confounded by the alterations in other nutrients (Weindruch et al. 1986; Bauerfeind and Fischer 2005). Furthermore, macronutrient sources, particularly protein, may contain a mixture of micronutrients or contain an unbalanced composition of amino acids. This is of particular concern in insect studies where hydrolysed yeast is the predominant protein source, as yeast is known to contain micronutrients, essential lipids, and carbohydrates (Tatar 2011). Although casein, a milk-derived protein extract, is used in many studies particularly within mammals (Fernandes et al. 1976), this source also contains carbohydrates, micronutrients and an unequal concentration of individual amino acids which confounds macronutrient effects (Jollès and Fiat 1979).

It is clear that the two aspects of diet, quantity and composition, are difficult to separate in practice and assess individually. Novel methods must be undertaken to pry apart these aspects and to successfully investigate the physiological responses to individual dietary components.

1.3.2 Pathogens and Immunity

The physiological consequences of pathogen interaction again should be considered as two aspects. The first derives from the immune activation of the host which incurs phenotypic changes in the organism, and the second is enforced by the pathogen itself. The majority of our understanding of immune activation has been through the experimentation of model organisms including insect species such as *D. melanogaster* (Lemaitre and Hoffman 2007; Kounatidis and Ligoxygakis 2012). The immune system of insects, although considered much simpler than our own, shares a surprising degree of similarity with that of mammals (Loker et al. 2004; Lemaitre and Hoffman 2007; Fauvarque and Williams 2011). Jawed vertebrates possess an adaptive immune system which employs specialised cell types, B and T lymphocytes, to produce specific antibodies and perform defined functions in the humoral and cell-mediated responses, respectively (Zimmerman et al. 2010). This complexity allows for antigenic ‘memory’ which is the basis of vaccination (Fearon and Locksley 1996). Another branch is innate immunity, which is known to be more general, or ‘non-specific’. It is this arm which our species share with insects (Vilmos and Kurucz 1998).

The innate immune system of insects comprises of both cellular and humoral processes which are known to interact (Hoffman 1995; Elrod-Erickson et al. 2000). The cellular response involves specialised blood cells (haemocytes) which encapsulate or phagocytose invading cells, activate coagulation, and secrete anti-microbial peptides (AMPs; Fauvarque and Williams 2011). The humoral response on the other hand, includes the activation of protease cascades, such as melanisation (which confines parasites to hardened proteinaceous capsules involving the activation of phenoloxidase), coagulation and the secretion of AMPs into the haemolymph from the fat body (Lemaitre and Hoffman 2007). The humoral response incorporates both the Toll and IMD pathways which lead to the upregulation of AMPs. These pathways are known to be pathogen-specific such that the Toll pathway is activated in response to gram positive bacteria, fungi and viruses (Lemaitre et al. 1997; Zambon et al. 2005), while the IMD is activated by gram negative bacteria (Tanji et al. 2007).

This immune response alone is known to elicit physiological costs within the host increasing ageing rates and reducing future pathogen resistance (Allen and Little 2011; Pursall and Rolff 2011). However, whether these costs alter with diet availability, change

the nutritional demands of the host, or differ between the sexes are not well known. Furthermore, the consequences of live infection can be dramatic for both the reproduction and survival of the infected organism (Bonds 2006; Velando et al. 2006; Weil et al. 2006). Infected hosts can adopt a range of physiological strategies in an attempt to alleviate the fitness costs enforced by the pathogen. These include a fecundity compensation strategy, where reproduction increases with often a reduction in pathogen resistance, or a fecundity reduction strategy, where reproduction is reduced and immune performance is enhanced (Hurd 2001; Weil et al. 2006). The conditions under which these specific strategies are adopted are not fully understood and elicit further investigation.

An interesting phenomenon gaining support in recent years has been the elicitation of physiological benefits without associated costs from the exposure to inactive pathogens (Leroy et al. 2012; Papp et al. 2012). This observation escapes the traditional physiological 'trade-offs' which are expected in phenotypic plasticity and has thus far remained unexplained (Forbes 2000; Agrawal et al. 2010). It is possible that these benefits result from the acute activation of stress response pathways as seen in hormetic treatments, however further investigation must be undertaken to identify whether this is the case (Gems and Partridge 2008). Determining the physiological consequences of immune activation and pathogen exposure, and how these vary between the sexes and with diet is important for our understanding of the underlying mechanisms of phenotypic plasticity and how organisms alleviate the costs of pathogen interaction in nature.

1.4 Thesis Contributions

My thesis investigates the patterns of the physiological response to environmental factors particularly diet and pathogen exposure, and explores how these may vary between the sexes. Throughout my thesis I have used *Drosophila melanogaster* as an investigative species, and *Metarhizium robertsii* to assess the effects of infection and pathogen exposure on the host's phenotype.

1.4.1 Use of *Drosophila-Metarhizium* System

Having previously established *D. melanogaster* as a model organism for assessing sex-specific physiological responses and phenotypic plasticity experienced to various conditions, we must understand why *M. robertsii* is a model species to investigate host-pathogen interactions. *Metarhizium* is a soil-borne, entomopathogenic fungus which is common to natural environments inhabited by the fruit fly (St Leger 2008). Although primarily known as a biological pest control agent, entomopathogenic fungi, such as *Beauveria bassiana* and *M. robertsii* (previously identified as *M. anisopliae* strain ARSEF 2575; Bischoff et al. 2009) are used to assess insect host-pathogen interactions due to their unique mode of infection (Clarkson and Charnley 1996). In this process, conidia attach to the epicuticle of the insect which germinate, penetrating through the cuticle with the secretion of digestive enzymes (Goettel et al. 1989). Following successful entry to the haemolymph, the fungal bodies proliferate immediately as protoplasts before producing blastospores and hyphae. Toxins are also produced suppressing the immune capacity of the host (Hunt and Charnley 2011).

The immunological response of insects to the mode of fungal infection is equally well understood. *Metarhizium* infection results in activation of the Toll pathway of the immune response from the identification of blastospores in the haemolymph leading to the upregulation of AMPs such as *Drosomycin* and *Metchnikowin* (Levitin and Whiteway 2008; Moret and Moreau 2012). Furthermore, a cellular-based response is elicited producing haemocytes which aid in the engulfment and melanisation of the invading cells (Lemaitre and Hoffman 2007). Aside from the detailed knowledge of the infection process, the ability to culture free-living colonies of *M. robertsii* in laboratory environments and the harvesting of viable spores enables their wide use in host-pathogen research. An example of such work is their use in understanding social

immunisation of eusocial species such as the ant (*Lasius neglectus*; Konrad et al. 2012). From the information collated for both organisms, the natural infection system, the ease of culturing and the tools available for both genetic and environmental manipulation, the *Drosophila-Metarhizium* host-pathogen model system shows great promise for providing insights into immunology, physiology and host-pathogen coevolution.

1.4.2 Development of the Holidic Diet in a Geometric Framework

To investigate the physiological responses of organisms to individual dietary components, I use holidic (chemically-defined) diets which are either presented simply in various protein:carbohydrate ratios, or within a geometric framework. Holidic diets are artificially produced food sources whereby the exact composition of nutrients is known (i.e. carbohydrates, amino acids, lipids, micronutrients and overall calories; Piper et al. 2014). The use of holidic diets eliminates potential fluctuations in the dietary components which aren't of interest (Tatar 2014). Therefore the addition of individual amino acids and the use of a single carbohydrate source, such as sucrose, to a fixed mixture of salts, vitamins, cholesterol and RNA provides researchers the precision required to assess the individual effects of macro & micro-nutrients and overall calories without potential noise from other constituents.

The geometric framework of nutritional manipulation provides the ability to separate the overall level of ingestion and the dietary components of a given diet. Termed nutritional geometry, this conceptual tool measures the intake of multiple nutrients (n), usually protein and carbohydrate, in n -dimensional nutritional space by comparing the level of ingestion of organisms conditioned to an array of diets that differ in both nutrient composition and concentration (Raubenheimer and Simpson 1999; Archer et al. 2009). Employing this method of dietary manipulation enables researchers to measure the exact quantities ingested by organisms in their entire lifetimes and to eliminate any potential noise from micronutrient fluctuations between diets (Lee et al. 2008). Therefore nutritional geometry dietary provides a tool with which to partition the effects of specific macronutrients and calories on phenotypic traits.

As this method commonly covers a large proportion of nutritional space in both macronutrient ratio and calorie content, as well as exact measurements of consumption, nutritional geometry is an exceptional system for assessing diet preference in organisms

experiencing varying conditions (e.g. sex and immune status). Investigations employing this method have identified how flies and crickets balance between their nutrient optima for lifespan and reproduction, as well as the dietary preferences of the sexes (Maklakov et al. 2008; Lee et al. 2008). Although this method has proved promising, these results have remained limited in their ability to identify the effects of individual nutrients as complex protein sources, such as casein and yeast, have been used (Tatar 2014).

Despite the advent of holidic diets and nutritional geometry, and the power which these methodologies hold in the field of nutritional science, no study has married these two concepts. It can be easily hypothesised that these systems can be applied to gain greater precision and understanding in how organisms respond to specific dietary components, and how theses differ between the sexes and with immunological status.

1.4.3 Thesis Overview

My thesis comprises of four data chapters (Chapters 2 – 5) each of which investigate the effect of a particular factor (or interaction between factors) on specific or various traits to better understand the plasticity of the physiological response.

In **Chapter 2** I address the questions of how the sexes respond differently to diet and whether they have sex-specific nutritional preferences. In collaboration with Dr. John Hunt and Dr. Kim Jensen from the University of Exeter, I use nutritional geometry to assess the dietary requirements of each sex for both lifespan and fecundity under control conditions. Additionally males and females are given a choice of diets to assess whether they manipulate their nutritional intake to obtain the resources required to maximise their fitness.

Chapter 3 investigates how the sexes vary in their response to pathogen infection and how this changes with diet. Using isocaloric diets which vary in protein:carbohydrate ratio and likelihood mortality modelling, I assess the characteristics of mortality which are affected across multiple diets as well as the diet-specific responses in survival of males and females exposed to live and inactive fungal spores. Furthermore I identify the pathogen load of these organisms to shed light on the tolerance and resistance of flies between diets and sexes. This study aims to highlight sex-specific variations in nutritional requirements for ageing and immune performance.

Chapter 4 aims to elucidate whether animals can manipulate their environment to maximise their fitness in response to environmental stress. Again in collaboration with Dr. Hunt and Dr. Jensen, and employing a nutritional geometry approach, I identify how diet is utilised by female flies between reproduction, ageing and pathogen resistance in response to pathogen challenge (exposure to heat-killed fungal spores) and live infection. Furthermore, I investigate whether flies adjust their dietary intake when exposed to these immunogenic treatments. The implications of this chapter therefore are to identify the nutritional costs of infection and immune deployment, and whether flies modify their dietary consumption to restrict the fitness impacts of infection.

In **Chapter 5** I address whether a particular environmental stress which confers physiological benefits (i.e. hormesis) incur consequences in other traits. I assess whether there is a link between the immune and stress pathways which underlie hormesis and further identify whether there are physiological trade-offs in the response. With the use of RNAi lines, I investigate the role of immune and stress genes in hormesis. Additionally, using outbred wild-type lines, I investigate the effects of the treatment on healthy ageing, fecundity and pathogen resistance to identify whether hormesis is an artefact of inbreeding. The implications of this chapter are therefore to identify whether hormesis is driven by the immune system, whether additional physiological costs are incurred, and thus whether it is a suitable treatment to improve human health.

Chapter 6 is a general discussion which highlights the findings of each data chapter, their potential implications for the fields of physiology and life history as well as their limitations. Furthermore, future projects are suggested with some promising avenues of research which are touched on from the conclusions of my chapters.

1.4.4 Supplementary Chapters

Additional to the main body of the thesis, my **Supplementary Chapters (SI & SII)** contain work of which I have contributed to throughout my PhD. These include:

SI – My first supplementary chapter tackles the same question addressed in Chapter 4, i.e. whether animals manipulate their environment to alleviate physiological and fitness costs in response to infection. In this work, I contribute to the investigation of whether flies alter their temperature preference when facing fungal infection. Aside from a behavioural assay, we assess the survival and reproductive consequences of infected

and uninfected flies when conditioned to temperatures chosen by the treated and untreated flies (22°C and 25°C respectively). This research has implications for the field of ecological immunity as it assists in understanding how animals use environmental variation in their response to pathogens which may assist in alleviating the costs of infection.

SII – The data presented in the second supplementary chapter aims to elucidate whether animals upregulate immune responses in anticipation of infection through sexual encounters. In collaboration with Dr. Elina Immonen and Prof. Mike Ritchie from the University of St. Andrew's, we assessed the effects of a stress gene with immune properties, *Turandot M* (*TotM*) a gene known to be upregulated in female flies prior to mating, in the defence of sexually transmitted fungal infections. Survival of uninfected, directly infected and flies infected through sexual contact was assessed in wild-type and mutant knockdown lines with suppressed expression levels of a number of immune-related genes. From this analysis, we could identify the effects of *TotM* in the resistance of sexually-transmitted infections.

1.5 References

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Sex-specific effects of protein and carbohydrate intake on reproduction but not lifespan in *Drosophila melanogaster*

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2.1 Abstract

Modest dietary restriction extends lifespan (LS) in a diverse range of taxa, and typically has a larger effect in females than males. Traditionally, this has been attributed to a stronger trade-off between LS and reproduction in females than males that is mediated by the intake of calories. Recent studies, however, suggest that it is the intake of specific nutrients that extends LS and mediates this trade-off. Here, we used the Geometric Framework (GF) to examine the sex-specific effects of protein (P) and carbohydrate (C) intake on LS and reproduction in *Drosophila melanogaster*. We found that LS was maximised at a high intake of C and a low intake of P in both sexes, whereas nutrient intake had divergent effects on reproduction. Male offspring production rate and LS were maximised at the same intake of nutrients, whereas female egg production rate was maximised at a high intake of diets with a P:C ratio of 1:2. This resulted in a stronger trade-off between LS and reproduction in females than males, as well as an optimal intake of nutrients for lifetime reproduction that differed across the sexes. Under dietary choice, the sexes followed similar feeding trajectories regulated around a P:C ratio of 1:4. Consequently, neither sex reached their nutritional optimum for lifetime reproduction suggesting intralocus sexual conflict over nutrient optimization. Our study shows clear sex differences in the nutritional requirements of reproduction in *D. melanogaster* and joins the growing list of studies challenging the role of caloric restriction in extending LS.

Running Title: Sex-specific nutritional trade-offs in fitness

Keywords: Geometric Framework, caloric restriction, protein, carbohydrate, lifespan, reproduction, fitness, nutrient regulation.

2.2 Introduction

Modest dietary restriction (DR, a reduction in food intake without malnutrition) has been shown to extend lifespan (LS) across a diverse array of taxa (Mair and Dillin 2008; Nakagawa et al. 2012) and this effect is typically more pronounced in females than males (Nakagawa et al. 2012). Traditionally, the effects of DR on LS have been attributed to caloric restriction (CR, Masoro 2002, 2005; Partridge and Brand 2005) and the observed sex differences explained by divergence in the energetic costs of reproduction (Barnes and Partridge 2003; Bonduriansky et al. 2008). In females, the extension of LS with CR is typically explained by the associated reduction in fecundity (Chapman and Partridge 1996) enabling greater investment in somatic maintenance (Partridge et al. 2005). In contrast, the energetic demands of reproduction in males are generally considered to be much lower so that the trade-off between LS and reproduction is less pronounced (Bonduriansky et al. 2008). Recent studies, however, have directly challenged these longstanding views by showing that it is the intake of specific nutrients and not calories *per se* that mediates this trade-off and are responsible for extending LS (Lee et al. 2008; Maklakov et al. 2008; Fanson et al. 2009; Fanson and Taylor 2012). Consequently, distinguishing between caloric and nutrient effects on LS and reproduction is an essential first step in understanding the mechanisms underlying LS and ageing in the sexes, yet one that continues to be the focus of much debate (Simpson and Raubenheimer 2007, 2009; Tatar, 2011; Piper et al. 2011; Fanson and Taylor 2012; Tatar et al. 2014).

The fruit fly model, *Drosophila melanogaster*, has played a central role in determining the relative importance of calories and nutrients in LS (Tatar 2011; Piper et al. 2011; Tatar et al. 2014). Early DR studies on *D. melanogaster* focused almost exclusively on the role of calories in extending LS (Chapman and Partridge 1996; Masoro 2002, 2005; Partridge and Brand 2005), although it is now clear that many of these studies confounded the effects of calories with those of specific nutrients (Tatar 2011; Piper et al. 2011; Tatar et al. 2014). More recent work has targeted the specific nutrients that extend LS and there is good evidence to suggest that protein (P) restriction is largely responsible for the extension in LS observed in many DR studies (Mair et al. 2005, Min and Tatar 2006, Piper et al. 2005; Lee et al. 2008, Nakagawa et al. 2012). However, despite considerable research we still know surprisingly little about the exact nutrient (or combination of nutrients) that extends LS in *D. melanogaster* (Tatar et al. 2014). A major

obstacle in addressing these questions has been the large diversity of approaches used to restrict dietary intake in this species (Tatar 2007, 2011; Lee et al. 2008; Piper et al. 2011; Tatar et al. 2014). The most common way to implement DR in *D. melanogaster* has been to restrict the intake of a diet of fixed composition by either limiting access to food or diluting the diet with water or another bulking agent and studies using this approach typically only use a small number of diets, do not precisely measure the intake of diet by and use yeast as a source of P or a way to alter overall caloric content (Tatar 2007; Piper et al. 2011). This approach can be problematic for a number of reasons. First, using too few diets can make it difficult (if not impossible) to adequately partition the effects of calories and nutrients on LS (Piper et al. 2005, 2011; Simpson and Raubenheimer 2007, 2009; Tatar 2011). Second, not measuring how much flies actually eat across their lifetime ignores compensatory feeding which can mask any effects of calories or nutrients on LS (Fanson et al. 2009). Finally, although P is the most abundant macronutrient in yeast, there is also a variety of micronutrients, essential lipids and sterols as well as carbohydrates (Simpson & Raubenheimer 2009; Tatar 2011). Thus, while P is the most likely nutrient regulating LS in DR studies using yeast (Mair et al. 2005, Min and Tatar 2006; Lee et al. 2008; Skorupa et al. 2008), any effects of P are necessarily confounded with these other constituents.

A solution to the above problems is provided by implementing the Geometric Framework (GF) approach using chemically-defined (holidic) diets. The GF is a conceptual tool that measures the intake of multiple nutrients (n) in n -dimensional nutritional space by constructing an array of diets that differ in both nutrient composition and concentration (Raubenheimer and Simpson 1999). As such, the GF provides a powerful way to partition the effects of nutrients and calories on LS (Simpson and Raubenheimer 2007, 2009). Lee et al. (2008) recently used this approach to show that it was the balance between the intake of P and C rather than calories *per se* that regulates reproduction and LS in female *D. melanogaster*, although hydrolyzed yeast was used as the source of P in this study. However, no study has yet fully integrated the GF with holidic diets to partition the effects of specific nutrients and calories on LS and reproduction in *D. melanogaster*, even though holidic diets exist and have proven success for this (Lee and Micchelli 2013; Piper et al. 2014) and other species of fruit fly (Fanson and Taylor 2012). Furthermore, no study has combined the GF with holidic diets to compare the effects of nutrients and

calories on LS and reproduction in the sexes. This work not only confirmed that LS and reproduction are regulated by the intake of specific nutrients and not calories in *T. commodus*, but also demonstrated that the sexes have very different nutritional optima for these traits (Maklakov et al. 2008). While it is tempting to conclude from this study that nutrients that are good for male LS may be detrimental to female LS and *vice versa*, clearly more work is needed before this should be viewed as a widespread pattern.

Here, we use the GF approach to distinguish the effects of nutrient intake (P and C) from the intake of calories on LS and reproduction in male and female *D. melanogaster*. We conducted two experiments: a no-choice (Experiment 1) and a choice (Experiment 2) experiment. In Experiment 1, we restricted 443 male and 494 female *D. melanogaster* to one of 29 different holidic, liquid diets varying systematically in P and C content, as well as total nutrition. This experiment provided detailed nutritional landscapes describing the effects of P and C on LS and reproduction in males and females and enabled us to formally compare the nutritional optima for these traits across the sexes. In Experiment 2, 100 flies of each sex were provided with choice between alternate diets differing in P:C ratio and total nutrition. This experiment determines if flies actively regulate their intake of nutrients when presented with dietary choice and, if so, whether this regulated intake point differs across the sexes. These regulated intake points can then be mapped onto the nutritional landscapes from Experiment 1 to ascertain whether the regulation of nutrients shown by the sexes is optimal with regard to LS and reproduction.

2.3 Materials & Methods

Fly stock and maintenance

Flies originated from the Dahomey stock (provided by Stuart Wigby, University of Oxford) and were maintained with overlapping generations in two large population cages (1 x 1 x 1m) at 25°C under a 12:12 L:D photoperiod at a population size of approximately two thousand flies per cage. Flies were cultured on standard sugar-yeast medium (80 g oat semolina, 138g treacle, 16g yeast, 7.5g agar, 2g methyl paraben, 206mL propionic acid, and 21mL phosphoric acid in 1.6 liters of water). Flies were reared at a density of 45-55 larvae per vial (25 × 95mm) with flies from 8 vials per week contributing to each population cage. Our stock cultures of flies were maintained according to this protocol for 2 years prior to use in our experiment.

All experimental flies were reared following this same protocol and housed in individual vials on the day they eclosed to adulthood. Once individually housed, flies were randomly allocated to experiments and to individual diets (Experiment 1) or diet pairs (Experiment 2) within experiments.

Artificial liquid diets and measuring nutrient intake

A total of 29 artificial liquid diets (Figure S1, Table S1) were constructed that varied in protein (P) and carbohydrate (C), as well as total nutrition (P + C), using a modified version of the protocol outlined in Fanson and Taylor (2012). This produced diets along 7 discrete nutritional rails (0:1, 1:16, 1:8, 1:4, 1:2, 1:1, 2:1; Figure S1). Proteins consisted of 18 different free amino acids (Table S2) mixed according to the protocol outlined in Chang et al. (2001), whereas carbohydrates consisted of sucrose. All diets also contained a fixed amount of cholesterol (4.00g/L), RNA from yeast (10.00g/L), Vanderzant vitamin mixture (3.60g/L), Wesson salt mixture (10.00g/L), and methyl paraben (1.50g/L). Our artificial diets therefore differed from those used by Fanson and Taylor (2012) where micronutrients were provided in direct proportion to the amount of protein contained in the diets. Our artificial diets were designed to cover the same nutrient space as the yeast and sucrose based diets used by Lee et al. (2008) and the holidic diets used by Fanson and Taylor (2012). We also included a single diet containing no protein or carbohydrates (Diet 29, Table S1) to root our nutritional landscapes at the origin.

Liquid diets were provided in either a single (Experiment 1) or a pair (Experiment 2) of 5µL microcapillary tubes (Drummond Microcaps; Ja et al. 2007, Lee et al. 2008) and the consumption of diets measured over 3-day feeding intervals. For both sexes, microcapillary tubes were removed at 9am on the morning of the third day while male and female reproduction was assessed (see below) and replaced with freshly filled tubes the following morning. For each feeding period, the amount of diet in microcapillary tubes was measured to the nearest 0.25mm before and after each feeding period using a precision ruler (Lee et al. 2008). To control for the evaporation of diets, two microcapillary tubes per diet were established in individual vials during each feeding period and diet loss measured as outlined above. Control vials were maintained in the same way as experimental vials with the exception that they did not contain a fly. Total diet consumption was estimated in each feeding period by subtracting the volume of diet in tubes before and after feeding and then subtracting the average loss of diet due to evaporation estimated from the two control tubes from this value. Total diet consumption was converted to an intake of specific nutrients by multiplying this volume by the nutrient composition of the diet provided in Table S1. We placed a small square (2 x 2cm) of moistened black absorbant paper in the bottom of all vials to serve as an additional water source and also as an oviposition site for females (Lee et al. 2008). This paper was replaced every 3 days at feeding. This feeding regime ensured that males and females had the same access to food throughout the experiment.

Experiment 1: No dietary choice on seven nutritional rails at four concentrations

To characterise and compare the linear and nonlinear effect of P and C intake on LS and reproduction in male and female *D. melanogaster*, 18 flies of each sex were assigned at random on their day of eclosion to each of the 29 artificial diets. Individual flies were fed and reproduction assessed every 3 days over the duration of their lifetime. Starting at day 3, all flies were paired with a virgin, 3-day old mating partner for 24 hours taken at random from the stock culture. This was repeated across the lifetime of all experimental flies, with a new 3-day old virgin mating partner being used each time. Female reproduction was assessed by counting the number of eggs oviposited on the moistened absorbent paper. Male reproductive success was assessed in competition against a virgin, 3-day old male harboring a dominant eye shape mutation (Krüppel) that allowed us to

easily determine the number of offspring sired by our experimental males. The Krüppel mutation had been backcrossed into our Dahomey stock for 28 generations prior to use in our experiment. After 24 hours, the Krüppel male and female were removed and the female established in an individual vial containing 7ml of “jazz mix” diet (Fisher Scientific, UK) for 14 days, after which time, vials were frozen and offspring phenotyped and counted. The survival of all experimental flies was monitored daily to measure lifespan. As LS differed across diets, reproduction was both provided across the lifetime of flies (male lifetime offspring production and female lifetime egg production), as well as a daily measure (male offspring production rate and female egg production rate). The latter was calculated by dividing lifetime reproduction by lifespan. In total, we had data available on nutrient intake, lifespan and reproduction for 443 male and 494 female flies. Flies that died before their first mating or escaped during the course of the experiment were excluded from our analyses.

Statistical Analysis

We used a multivariate response-surface approach (Lande and Arnold 1983) to estimate the linear and nonlinear (i.e. quadratic and correlational) effects of P and C intake on our response variables (LS, lifetime and daily reproduction) for each sex. Nutritional landscapes were visualised using nonparametric thin-plate splines implemented in the *FIELDS* package of R (version 2.13.0). We used a sequential model building approach to assess whether the linear and nonlinear effects of protein and carbohydrate intake differed for our response variables within and across the sexes (Draper and John 1988; South et al. 2011). When significant linear (i.e. P and C) or quadratic (i.e. P x P and C x C) effects were detected in these sequential models, univariate tests were used to determine which of the nutrients were responsible (South et al. 2011). As our response variables were measured in different units, they were standardised using a Z-transformation prior to analysis (South et al. 2011).

Experiment 2: Measuring nutrient intake under dietary choice

To examine how male and female *D. melanogaster* regulate their intake of nutrients when provided dietary choice, 20 flies of each sex were assigned at random on their day of eclosion to each of 5 different diet pairs that vary in both the ratio of protein to

carbohydrates, as well as total nutrition (P:C(total nutrition)): pair 1: 1:1 (180g/L) vs 0:1 (180g/L), pair 2: 1:1 (180g/L) vs 0:1 (360g/L), pair 3: 1:1 (360g/L) vs 0:1 (180g/L), pair 4: 1:1 (360g/L) vs 0:1 (360g/L) and pair 5: 1:2 (360g/L) vs 0:1 (360g/L). This corresponds to diets 7, 8, 12, 27 and 28 in Table S1 and provides good coverage of nutrient space on the nutritional landscape (Figure S1). As outlined above, the consumption of both diets in each pair was measured every 3 days for 15 days post-eclosion and the same mating scheme was followed as in Experiment 1. In total, we had data available on nutrient intake under dietary choice for 66 males and 86 females. Flies that died before 15 days or escaped during the course of the experiment were excluded from our analyses.

Statistical analysis

To determine if flies consumed significantly more of one diet in each pair, we compared the total absolute consumption of both diets using paired *t*-tests. To determine the implications of dietary choice for nutrient intake, we first calculated the expected intake of P and C for each fly assuming they consumed diets at random. This expected intake was then subtracted from the observed intake of these nutrients and the difference compared to a mean of zero (i.e. expected of cockroaches were eating at random) using a one-sample *t*-test (South et al. 2011).

To determine if the intake of nutrients changed with diet pair and sex over the duration of our feeding experiment, we analyzed the cumulative intake of P and C across the 5 feeding periods using repeated-measures ANOVA. We included sex, diet pair and time as main effects in this model, plus all possible interaction terms. Significant interactions between sex and time and/or between sex, diet pair and time would indicate that the sexes follow different feeding trajectories over time. As this overall model showed significant differences in nutrient intake across diet pairs (Table S3), we conducted post-hoc analysis within each of the pairs using a reduced model that included only sex, time and their interaction. This same model was used to compare the average cumulative intake of nutrients across diet pairs.

The regulated intake point for each sex, defined as the point in nutritional space to which animals regulate when provided with dietary choice (Simpson et al. 2004), was calculated as the mean total intake of P and C across all diet pairs. To determine if the regulated intake point differed between the sexes we used ANCOVA including sex (main

effect), total P intake (covariate) and their interaction as model terms and total C intake as the response variable. A significant sex by protein intake interaction would indicate that the sexes have different regulated intake points. We also compared the regulated intake point for each sex to a P:C ratio of 1:4, by calculating and then subtracting the expected intake of nutrients by each fly if consuming nutrients at this ratio from the observed intake of nutrients and comparing this difference to a mean of zero using a one-sample *t*-test.

2.4 Results

Experiment 1: No dietary choice on seven nutritional rails at four concentrations

The nutritional landscapes show that LS and reproduction in *D. melanogaster* are heavily influenced by the intake of P and C (Figure 1). In both sexes, LS was maximised at a high intake of diets containing a low P:C ratio of 1:16 (Figures 1A & B; Table 1). With the exception of very low nutrient intake ($<50\mu\text{g/day}$), LS decreased with P intake across isocaloric lines on the nutritional landscapes (Figures 1A & B). Furthermore, LS increased with total caloric intake along each of the nutritional rails in our geometric design, although this was less pronounced as the P:C ratio of diets increased (Figures 1A & B). Consequently, these findings provide little support for the notion that caloric restriction extends LS in *D. melanogaster* but do highlight the key role that P and C intake plays in mediating LS (Lee et al. 2008; Maklakov et al. 2008; Fanson et al. 2009; Fanson and Taylor 2012). Formal statistical comparison showed little difference between the sexes in the effects of P and C intake on LS (Table 2).

Male offspring production rate was maximised at a high intake of diets containing a low P:C ratio (Figure 1C; Table 1) and therefore peaked in a similar region on the nutritional landscape as male LS. In contrast, female egg production rate was maximised at a high intake of more P rich diets, peaking at a P:C ratio of 1:2 (Figure 1D; Table 1). As a result, the nutritional landscapes for the daily rate of reproduction in males and females differed significantly (Table 2). Detailed inspection of the sequential models (Table 2) showed that this sex difference was driven by the linear and nonlinear effects of both nutrients on the daily rate of reproduction. We found qualitative similar effects of P and C intake on male lifetime offspring production and female lifetime egg production (Figures 1E & F, Table 1), as well as differences in these nutritional landscapes across the sexes (Table 2). One obvious exception, however, was that the difference in the linear effects of nutrient intake on lifetime reproduction in both sexes was due exclusively to P (Table 2).

The divergence in the nutritional requirements of the sexes also influences the magnitude of the trade-off between LS and reproduction within the sexes. In males, LS and measures of daily and lifetime reproduction can all be maximised on a high intake of diets with a low P:C ratio, providing little evidence for a trade-off between these traits. In fact, comparison of the nutritional landscapes for male lifespan and reproduction showed that only the linear component of these surfaces differed significantly due to the stronger

positive effects of high C intake on lifespan than daily and lifetime reproduction (Table 2). In females, however, the intake of nutrients that maximised lifespan resulted in suboptimal reproduction and *vice versa*. Consequently, there were significant differences in the linear and nonlinear components of the nutritional landscapes for female lifespan and measures of reproduction that resulted from the effects of both protein and carbohydrate intake (Table 2). Therefore in contrast to males, this finding demonstrates a clear trade-off between lifespan and reproduction in females.

Experiment 2: Measuring nutrient intake under dietary choice

When given dietary choice, both sexes showed a clear preference for the diet containing the highest concentration of carbohydrates on diet pairs 1 and 3, and females also expressed this preference on diet pair 4 (Figure S2). Importantly, for both sexes this resulted in a significantly higher intake of carbohydrates than expected if flies fed at random from diets for all diet pairs and a significantly lower intake of protein for diet pairs 1 and 3 for males and diet pairs 1, 3 and 4 for females (Figure S3).

There were clear effects of sex, diet pair and time on the cumulative intake of P and C: on average, females had a higher intake of both nutrients than males, flies consumed more nutrients on diet pairs 3, 4 and 5 than on diet pairs 1 and 2, and nutrient intake increased with time (Table S3; Figures 2A-E). There was also a significant interaction between diet pair and time for both nutrients as the cumulative intake trajectories were steeper on some diet pairs (e.g. diet pair 1) than others (e.g. diet pair 4) (Table S3; Figures 2A-E). Importantly, however, the interactions between sex and time and between sex, diet pair and time were not significant indicating that the sexes follow the same cumulative feeding trajectories over time (Table S3; Figures 2A-E). Consequently, the regulated intake point for males (P:C ratio, 1:4.43) and females (1:3.96) did not differ significantly (Sex: $F_{1,138} = 4.38$, $P = 0.038$; P intake: $F_{1,138} = 107.19$, $P = 0.0001$; Sex x P intake: $F_{1,138} = 0.08$, $P = 0.79$). Moreover, neither deviated significantly from a P:C ratio of 1:4 (males: $t_{61} = 1.90$, $P = 0.06$; females: $t_{79} = 0.28$, $P = 0.78$), which was shown to maximise lifetime reproductive success in female *D. melanogaster* (Figure 1; Lee et al. 2008). Although in close proximity, the regulated intake points for each sex did not completely reside on the nutritional peaks for LS or reproduction suggesting that dietary regulation is not optimal in *D. melanogaster* (Figure 1).

2.5 Discussion

It is widely accepted that modest DR extends LS, having been shown in a wide diversity of species, ranging from yeast to primates (Mair and Dillan 2008), and that this effect is typically stronger in females than in males (Nakagawa et al. 2012). Traditionally, the effects of DR on LS have been attributed to CR (Masoro 2002, 2005; Partridge and Brand 2005) and the stronger effect observed in females due to the greater energetic costs of reproduction (Barnes and Partridge 2003; Bonduriansky et al. 2008). Here we provide empirical evidence that directly contradicts this traditional view. We found that both LS and reproduction in male and female *D. melanogaster*, as well as the trade-off between these two traits in the sexes, is primarily determined by the intake of P and C rather than the intake of calories. Thus, our work adds to the growing list of studies challenging a central role for calories in extending LS (Mair et al. 2005; Lee et al. 2008; Maklakov et al. 2008; Skorupa et al. 2008; Fanson et al. 2009; Ja et al. 2009; Fanson and Taylor 2012; Nakagawa et al. 2012) and highlights the utility of applying a robust nutritional framework to disentangle the effects of nutrients and calories on LS (Simpson and Raubenheimer 2007, 2009).

We found that the intake of P and C had clear effects on LS in *D. melanogaster* and these effects were largely consistent across the sexes. In both sexes, LS was maximised at a high intake of nutrients at a P:C ratio of approximately 1:16 (Figures 1A & B) and, for a given caloric intake, decreased sharply as the P:C ratio of diets increased (i.e. became more P biased). Moreover, LS decreased with a reduced total intake of nutrients (and calories) in both sexes, especially on diets with a low P:C ratio. Our work is therefore consistent with previous studies showing that LS extension under DR in *D. melanogaster* is driven by a restricted intake of P and not calories *per se* (Mair et al. 2005; Skorupa et al. 2008; Lee et al. 2008; Ja et al. 2009; Tatar 2011). Our nutritional landscape for female LS (Figure 1B) shows a strong resemblance to an earlier study by Lee et al. (2008) on this species using the GF. Although yeast-based diets were used, Lee et al. (2008) also found that female LS was maximised at a high intake of nutrients at a P:C ratio of 1:16. Our landscape is also broadly similar to those constructed for female Queensland fruit flies (Q-flies, *Bactrocera tryoni*, Fanson et al. 2009; Fanson and Taylor 2012) and field crickets (*T. commodus*, Maklakov et al. 2008) that also show maximal LS on low P:C ratio diets. However, the exact P:C ratio maximising female LS in *T.commodus* is less C biased (P:C

ratio = 1:8, Maklakov et al. 2008) and *B. tryoni* is more C biased than shown for *D. melanogaster*, the magnitude depending on whether yeast-based (1:21, Fanson et al. 2009) or holidic (1:32, Fanson and Taylor 2012) diets were used. To date, only a single study by Maklakov *et al.* (2008) has compared the effects of P and C intake on LS across the sexes using the GF and found that male and female field crickets have different nutritional optima for LS. Although contrary to our findings for *D. melanogaster*, it is important to note that the sex difference shown for *T. commodus* resulted from LS declining at very high C intake in males but not in females rather than a large shift in the P:C ratio maximizing LS in the sexes (Maklakov et al. 2008). That is, male LS was maximised at P:C ratio of 1:5 but the peak on the nutritional landscape was broad and clearly overlapped the P:C ratio maximizing female LS (1:8) in this species (Maklakov et al. 2008). Collectively, these studies provide compelling support for the protein restriction hypothesis (Simpson and Raubenheimer 2007, 2009) and suggest that the extension of LS with P restriction may be a widespread pattern across the animal kingdom. Indeed a recent meta-analysis across DR studies showed that the effect of P intake on LS is much larger than that of caloric intake (Nakagawa et al. 2012).

In contrast to LS, we found strong divergence in the effects of P and C on reproduction across the sexes of *D. melanogaster* (Figures 1C & D). Male offspring production rate was maximised at the same P:C ratio as LS (1:8, Figure 1C), whereas female egg production was maximised at a high intake of diets with a P:C ratio of 1:2 (Figure 1D). This difference in the nutritional requirements for reproduction most likely reflects the divergence in the reproductive strategies of the sexes. In most species, the intensity of sexual selection acting on males is far greater than on females because fathers contribute less to each offspring than mothers do (Bonduriansky et al. 2008). As a result, males often face intense competition for access to mates and individuals with the most elaborate sexual traits or behaviors are frequently the most successful (Andersson 1994). To fuel these costly traits and behaviors, males require a high intake of C as this provides an abundant source of energy that can be accessed rapidly after digestion (Maklakov et al. 2008; South et al. 2011). For example, producing an advertisement call, a key determinant of mating success in male *T. commodus* (Hunt et al. 2004), is metabolically demanding (Kavanaugh 1987) and is maximised at a P:C ratio of 1:8 (Maklakov et al. 2008). Likewise, sex pheromone expression and subsequent

attractiveness is also maximised at a P:C ratio of 1:8 in male cockroaches (*Nauphoeta cinerea*; South et al. 2011). In contrast, females typically do not have to compete for matings and their reproductive success is largely determined by the number of eggs they produce. In many insect species, egg production is closely linked to nutrition through a neurohormonal feedback system and P intake plays an important role in stimulating oogenesis and regulating vitellogenesis (Wheller 1996). It is therefore not surprising that females typically require a higher intake of P than males to maximise their reproductive success (Maklakov et al. 2008). Indeed, our finding that the rate of egg production in *D. melanogaster* is maximised at a P:C ratio of 1:2 is highly consistent with previous studies incorporating a nutritional geometry (NG) approach on this species (P:C = 1:2, Lee et al. 2008; Reddiex et al. 2014), as well as female field crickets (1:1, Maklakov et al. 2008) and Q-flies (1:2.3, Fanson et al. 2009; 1:1, Fanson and Taylor 2012).

The sex difference we show in the effects of P and C intake on reproduction in *D. melanogaster* is also surprisingly consistent with the divergence observed in male and female field crickets (Maklakov et al. 2008). However, our findings contradict a recent study on *D. melanogaster* that showed male and female reproduction were both maximised on diets with a P:C ratio of 1:2 (Reddiex et al. 2013). Although male reproduction was measured as the number of offspring sired in competition in both studies, this was measured over the entire LS of each male in our study but only over the first four days of adulthood in Reddiex et al. (2013). In *D. melanogaster*, an intermediate intake of P over the first 12 days of adulthood provides males with an advantage in sperm competition, most likely through an increase in the production of sperm and/or Acps (Fricke et al. 2008). It is therefore possible that the difference between our study and Reddiex et al. (2013) reflects the greater need for P early in adulthood to promote sexual maturation. Indeed, the feeding data from our choice experiment shows that the P intake of males increases over the first 9 days of adulthood before levelling off, whereas C intake continued to increase over the 15 day feeding period (Figure S4).

The trade-off between reproduction and LS due to the competing demands for resources is central to evolutionary theories of ageing (Williams 1966; Kirkwood and Holliday 1979; Barnes and Partridge 2003; Partridge et al. 2005). Traditionally, calories have been viewed as the limiting resource that regulates this trade-off (Gadgil and Bossert 1970; Bell 1980) and the greater energetic demands of reproduction has been

used to explain why this trade-off is typically stronger in females than males (Barnes and Partridge 2003; Bonduriansky et al. 2008). In agreement with this view, we show that there is a trade-off between LS and reproduction in female but not male *D. melanogaster*. However, we show that this trade-off is mediated by the intake of P and C rather than calories. The nutritional landscapes for LS and reproduction in males were similar, with both traits being maximised on a high intake of diets with a low P:C ratio (Figures 1A & C). In contrast, female LS and reproduction were maximised at very different regions in nutrient space: LS increased with C intake across isocaloric rails on the nutritional landscape, whereas reproduction was maximised at an intermediate P:C ratio and declined as diets became more P or C biased (Figures 1B & D). Thus, the intake of nutrients that maximises LS in females is suboptimal for reproduction and *vice versa*. This finding is consistent with the classic Y-model of life-history trade-offs (van Noordwijk & De Jong 1986; de Jong 1993) which states that both LS and reproduction cannot be maximised because increasing reproductive effort diverts essential resources away from somatic maintenance and LS.

Alternatively, the observed differences in the nutritional landscapes for LS and reproduction in females may arise because there are direct costs to P ingestion (Simpson and Raubenheimer 2009; Fanson et al. 2012). The intake of P is clearly required for egg production in *D. melanogaster* but above a certain intake, P has a detrimental effect on LS and reproduction. Importantly, this pattern was also shown in males suggesting this effect is not contingent on the allocation of resources to reproduction *per se* but rather a direct consequence of over-ingesting this nutrient. The most likely candidates responsible for this effect are the elevated production of toxic nitrogenous wastes (Singer 2003) or mitochondrial reactive oxygen species that are known to increase with P consumption (Sanz et al. 2004; Ayala et al. 2007). In general, there is growing support for the lethal P hypothesis (Simpson and Raubenheimer 2009; Fanson et al. 2012) with similar declines in LS and reproduction at high P intake being reported in all species where the NG approach has been used (Maklakov et al. 2008; Lee et al. 2008; Fanson et al. 2009; Fanson and Taylor 2012). Moreover, Fanson et al. (2012) recently provided direct empirical support for this hypothesis by showing that Q-flies varying in reproductive status (mated, virgin and sterilised females and virgin males) all experienced a similar decrease in LS with increasing P intake, even though sterilised females and males require little P for

reproduction. Further tests of this nature are needed to separate the relative importance of the Y-model and lethal P hypothesis to the trade-off between LS and reproduction in *D. melanogaster*.

Optimal foraging theory predicts that animals will evolve foraging mechanisms that maximises their fitness (MacArthur and Pianka 1966). While early studies focused exclusively on the intake of energy (Stephens and Krebs 1986), there is now considerable evidence demonstrating that animals can also regulate their intake of specific nutrients to maximise fitness (e.g. Simpson et al. 2004, 2006; Dussutour & Simpson 2009; Jensen et al. 2012). We found that our best estimate of fitness (lifetime reproduction) was highly divergent across the sexes in *D. melanogaster* suggesting that fitness will be best maximised by the sexes regulating their intake of P and C independently. Despite this, we failed to see divergence in the feeding trajectories of the sexes under dietary choice with males and females both regulating their intake of nutrients at a P:C ratio of 1:4 (Figure 2F). While comparable with earlier studies on female *D. melanogaster* (P:C = 1:4, Lee et al. 2008) and Q-flies (1:3, Fanson et al. 2009), this pattern of nutrient regulation was not optimal for either sex (Figures 1E & F). Furthermore, the lack of divergence between the sexes is consistent with the pattern shown in field crickets where there is clear sex-specific nutritional optima for lifetime reproduction but the sexes shared a common feeding trajectory at a P:C ratio of 1:2.96 (Maklakov et al. 2008). Maklakov et al. (2008) argued that this shared dietary choice prevents the sexes from reaching their sex-specific nutrient optima: a process referred to as intralocus sexual conflict (ISC; Bonduriansky and Chenoweth 2009). ISC arises whenever there are sex-specific optima for a trait that is expressed in both sexes but the shared genetic basis for this trait prevents the sexes from evolving independently to their optima (Bonduriansky and Chenoweth 2009). Definitive evidence of ISC over nutrient optimisation therefore requires showing that the sexes (i) have different nutritional optima and (ii) share a common genetic basis for their dietary preferences, which can be characterised by a strong and positive intersexual genetic correlation (r_{MF} ; Bonduriansky and Chenoweth 2009). To date, only a single study has measured these key parameters and concluded there was little evidence for ISC over nutrient optimization in *D. melanogaster* (Reddiex et al. 2013). This result, however, is not altogether unsurprising given that nutrient intake was only measured over a very short time period (4 days) which is likely to explain the minor differences in the nutritional

landscapes across the sexes (relative to the differences we show) and a r_{MF} for P intake that did not differ statistically from zero (Reddiex et al. 2013). Like the study of Maklakov *et al.* (2008), our results are highly suggestive that ISC over nutrient optimization exists in *D. melanogaster* but further work is needed to estimate r_{MF} over an appropriate timeframe before this process can be viewed as an important evolutionary constraint in this species.

Acknowledgements

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2.7 Figures & Tables

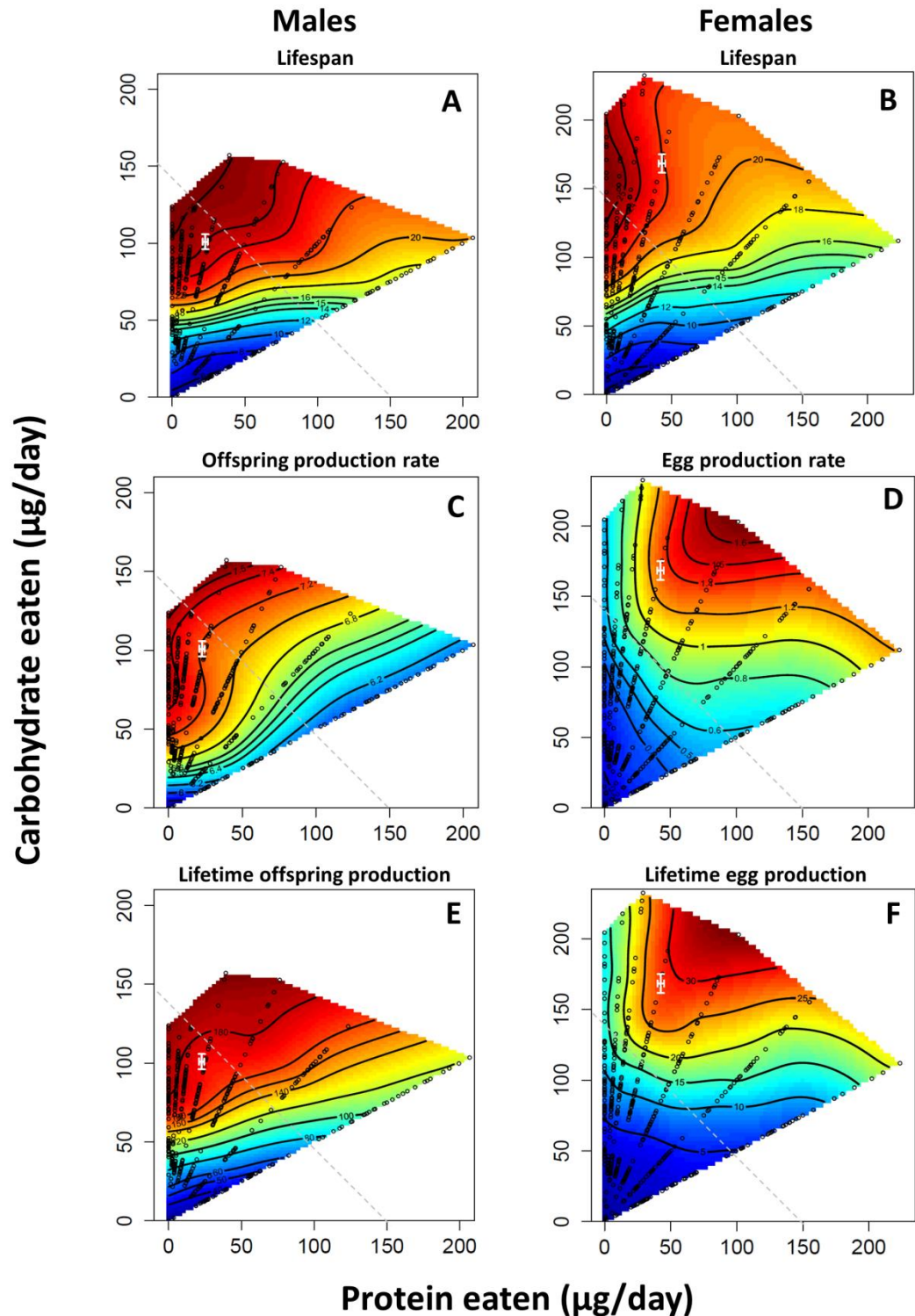


Figure 1: Nonparametric thin-plate spline contour visualizations of the responses surfaces describing the effects of protein and carbohydrate intake on **A** male lifespan, **B** female lifespan, **C** male offspring production rate, **D** female egg production rate, **E** Lifetime offspring production in males and **F** lifetime egg production in female *Drosophila melanogaster*. Individual flies were allowed to feed *ad libitum* from one of the 29 liquid

diets across their adult lifespan and open black circles represent the intake of protein and carbohydrates along each of the 7 nutritional rails by individual flies. The regulated intake point (\pm SE) for flies given the choice between alternate diets (Experiment 2) is mapped on each landscape (in white), after being converted to a daily intake. On each landscape, the grey dashed line represents an “isocaloric line” across the nutritional landscape where a given intake of nutrients yields equal calories. As protein and carbohydrates contain approximately the same calories per unit ingested (~ 4 calories/g), the slope of the isocaloric line is -1.

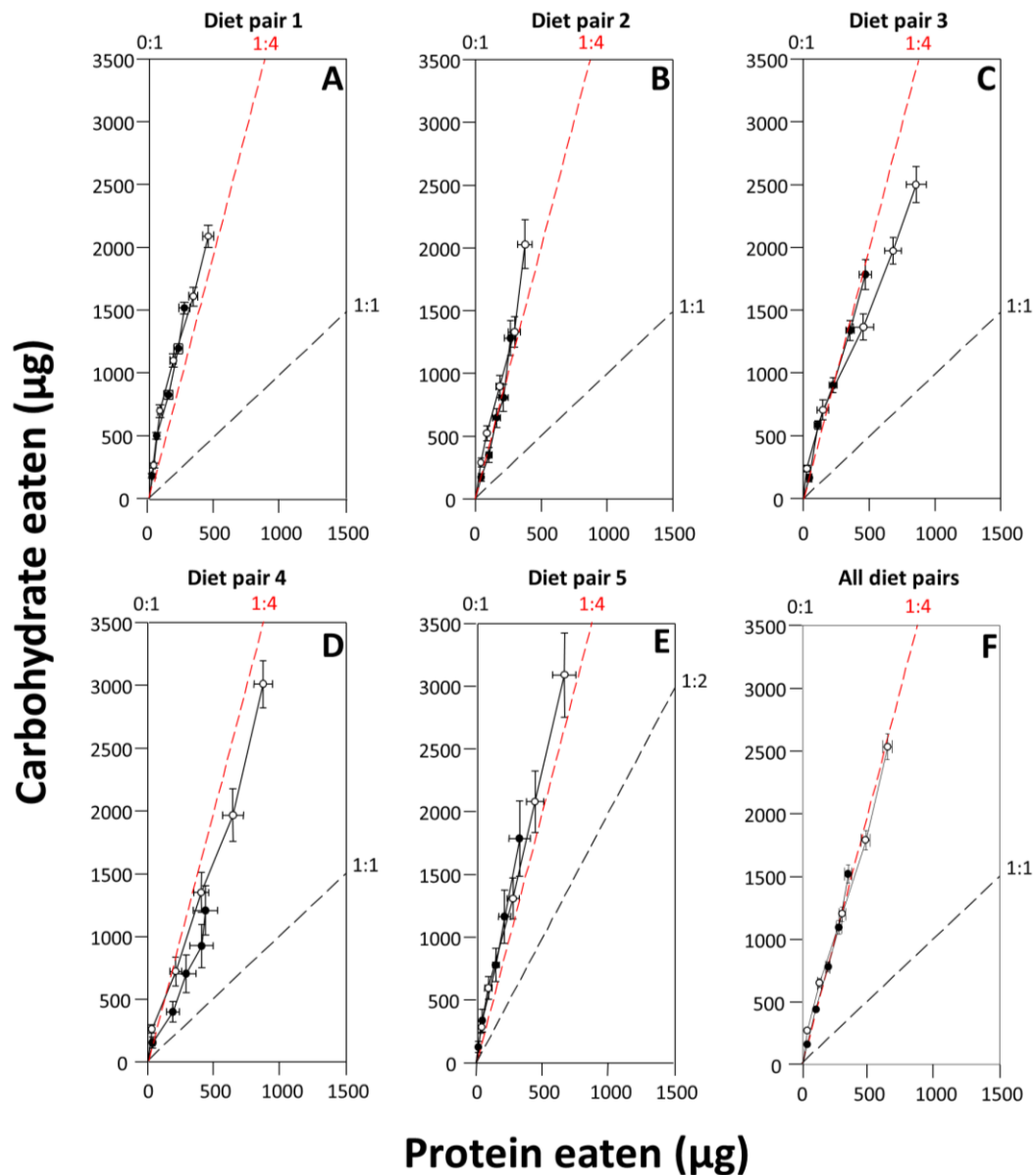


Figure 2: Cumulative intake of protein and carbohydrates (mean \pm SE) by male (closed symbols) and female (open symbols) *Drosophila melanogaster* over the first 15 days of adulthood when given the choice between five different diet pairs: **A** diet pair 1: diet 7 versus diet 27; **B** diet pair 2: diet 7 versus diet 28; **C** diet pair 3: diet 8 versus diet 27; **D** diet pair 4: diet 8 versus diet 28; **E** diet pair 5: diet 12 versus diet 28 (see Table S1 for diet compositions). **F** The average cumulative intake of protein and carbohydrates across all diet pairs. The terminal feeding points in **F** represent the regulated intake point for each sex. The red dashed line represents the cumulative intake of nutrients if flies consumed these nutrients in a P:C ratio of 1:4.

Table 1: Linear and nonlinear effects of protein (P) and carbohydrate (C) intake on lifespan and reproduction in male and female *Drosophila melanogaster*.

Response variable	Linear effects		Nonlinear effects		
	P	C	P × P	C × C	P × C
Males					
<i>Lifespan</i>					
Gradient ± SE	-0.18 ± 0.03	0.80 ± 0.03	0.03 ± 0.02	-0.17 ± 0.03	0.03 ± 0.05
t_{442}	5.6	25.4	1.1	5.8	0.5
<i>P</i>	0.001	0.001	0.28	0.001	0.60
<i>Offspring production rate</i>					
Gradient ± SE	-0.11 ± 0.05	0.13 ± 0.05	-0.01 ± 0.04	-0.11 ± 0.04	0.06 ± 0.08
t_{442}	2.3	2.7	0.2	2.4	0.7
<i>P</i>	0.02	0.007	0.81	0.02	0.50
<i>Lifetime offspring production</i>					
Gradient ± SE	-0.21 ± 0.04	0.67 ± 0.04	0.01 ± 0.03	-0.18 ± 0.04	0.07 ± 0.06
t_{442}	5.5	17.7	0.4	5.0	1.1
<i>P</i>	0.001	0.001	0.72	0.001	0.26
Females					
<i>Lifespan</i>					
Gradient ± SE	-0.18 ± 0.03	0.74 ± 0.03	0.06 ± 0.02	-0.21 ± 0.02	-0.03 ± 0.04
t_{493}	5.7	23.3	3.0	9.3	0.7
<i>P</i>	0.001	0.001	0.003	0.001	0.46
<i>Egg production rate</i>					
Gradient ± SE	0.26 ± 0.04	0.38 ± 0.04	-0.13 ± 0.03	-0.01 ± 0.03	0.19 ± 0.05
t_{493}	6.5	9.5	4.4	0.5	3.6
<i>P</i>	0.001	0.001	0.0001	0.65	0.001
<i>Lifetime egg production</i>					
Gradient ± SE	0.12 ± 0.04	0.60 ± 0.04	-0.10 ± 0.03	-0.03 ± 0.03	0.23 ± 0.05
t_{493}	3.4	16.9	3.7	1.0	4.8
<i>P</i>	0.001	0.001	0.001	0.33	0.001

Table 2: Sequential *F*-tests comparing the effects of protein and carbohydrate intake on lifespan and reproduction within and between the sexes in *Drosophila melanogaster*. The sequential *F*-tests test the differences in the sign and strength of the linear, quadratic and correlational regression gradients across different response variables or the sexes. When significant differences in linear or quadratic regression gradients were detected, univariate tests were used to determine whether this overall effect was due to the intake of protein or carbohydrates (or both).

	<i>SS_R</i>	<i>SS_C</i>	<i>DF₁</i>	<i>DF₂</i>	<i>F</i>	<i>P</i>
Males vs. Females						
<i>Lifespan vs. Lifespan</i>						
Linear	413	413	2	931	1.0	0.36
Quadratic	356	355	2	927	2.1	0.12
Correlational	355	355	1	925	0.8	0.39
<i>Offspring production rate vs. Egg production rate</i>						
Linear	860	804	2	931	32.3	0.001 ^A
Quadratic	798	788	2	927	5.5	0.004 ^B
Correlational	780	774	1	925	7.2	0.01
<i>Lifetime offspring production vs. Lifetime egg production</i>						
Linear	577	553	2	931	20.2	0.001 ^C
Quadratic	546	534	2	927	11.0	0.001 ^D
Correlational	522	520	1	925	4.0	0.05
Males						
<i>Lifespan vs. Offspring production rate</i>						
Linear	705	611	2	880	67.5	0.001 ^E
Quadratic	591	589	2	876	1.2	0.30
Correlational	589	589	1	874	0.1	0.77
<i>Lifespan vs. Lifetime offspring production</i>						
Linear	441	437	2	880	4.4	0.01 ^F
Quadratic	405	405	2	876	0.0	0.97
Correlational	404	404	1	874	0.3	0.59
Females						
<i>Lifespan vs. Egg production rate</i>						
Linear	670	605	2	982	52.5	0.001 ^G
Quadratic	588	554	2	978	30.0	0.001 ^H

Correlational	551	544	1	976	11.2	0.001
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Lifespan vs. Lifetime egg production

Linear	552	529	2	982	21.6	0.001 ^I
Quadratic	511	484	2	978	27.6	0.001 ^J
Correlational	479	470	1	976	17.2	0.001

Univariate tests: ^A = P: $F_{1,931} = 35.5$, $P = 0.0001$, C: $F_{1,931} = 15.6$, $P = 0.0001$; ^B = PxP: $F_{1,927} = 5.7$, $P = 0.017$, CxC: $F_{1,927} = 4.5$, $P = 0.034$; ^C = P: $F_{1,931} = 40.4$, $P = 0.0001$, C: $F_{1,931} = 1.7$, $P = 0.19$; ^D = PxP: $F_{1,927} = 6.3$, $P = 0.013$, CxC: $F_{1,927} = 14.4$, $P = 0.0001$; ^E = P: $F_{1,880} = 1.2$, $P = 0.28$, C: $F_{1,880} = 131.3$, $P = 0.0001$; ^F = P: $F_{1,880} = 0.4$, $P = 0.54$, C: $F_{1,880} = 7.0$, $P = 0.008$; ^G = P: $F_{1,982} = 73.9$, $P = 0.0001$, C: $F_{1,982} = 49.0$, $P = 0.0001$; ^H = PxP: $F_{1,978} = 22.1$, $P = 0.0001$, CxC: $F_{1,978} = 35.6$, $P = 0.0001$; ^I = P: $F_{1,982} = 40.1$, $P = 0.0001$, C: $F_{1,982} = 8.2$, $P = 0.004$; ^J = PxP: $F_{1,978} = 15.3$, $P = 0.0001$, CxC: $F_{1,978} = 37.8$, $P = 0.0001$;

2.8 Supplementary Figures & Tables

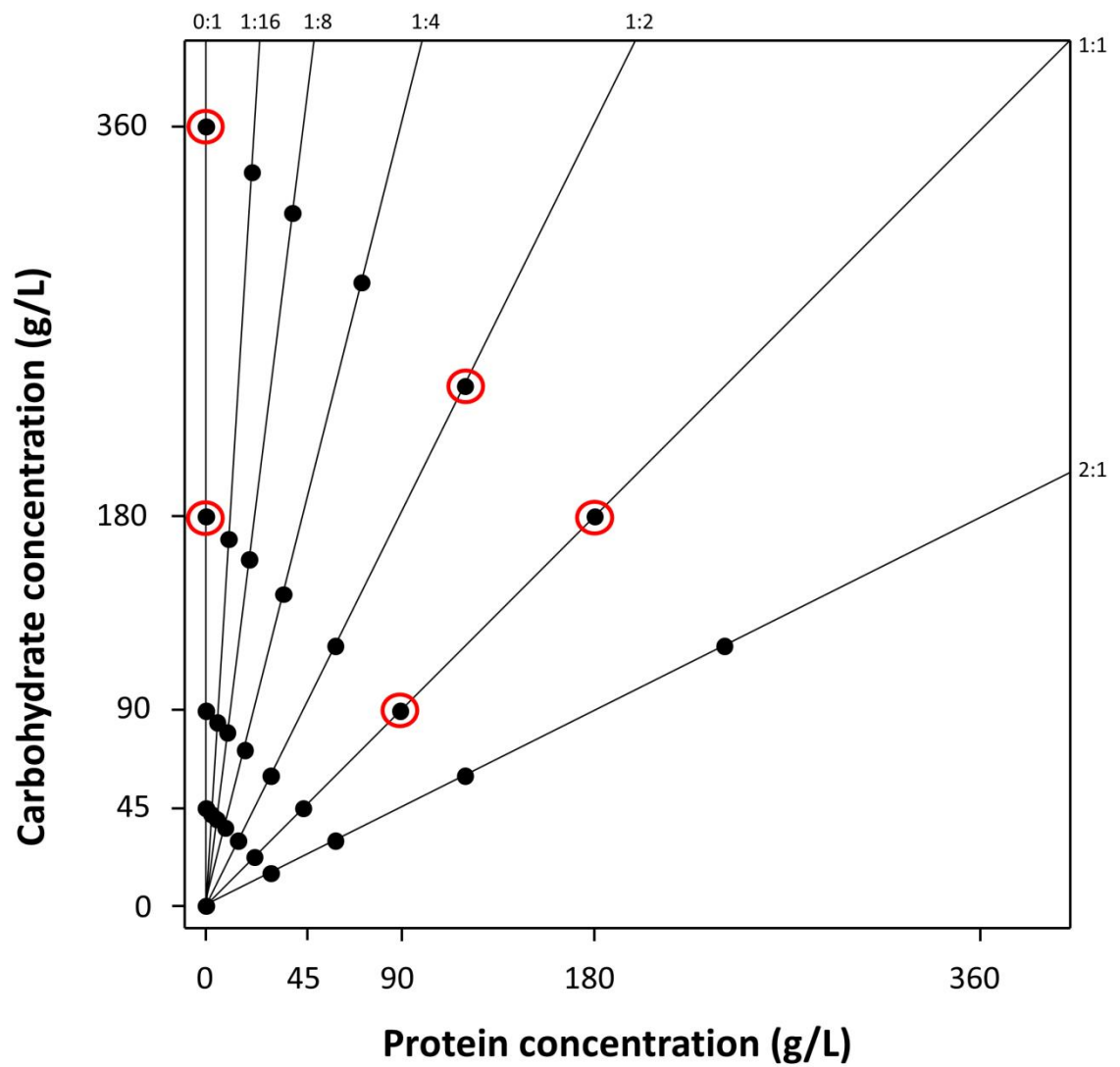


Figure S1: Geometric presentation of the protein and carbohydrate concentration in the 29 artificial diets used in our no-choice experiment (Experiment 1). The individual diets circled represent the five diets used in our dietary choice experiment (Experiment 2).

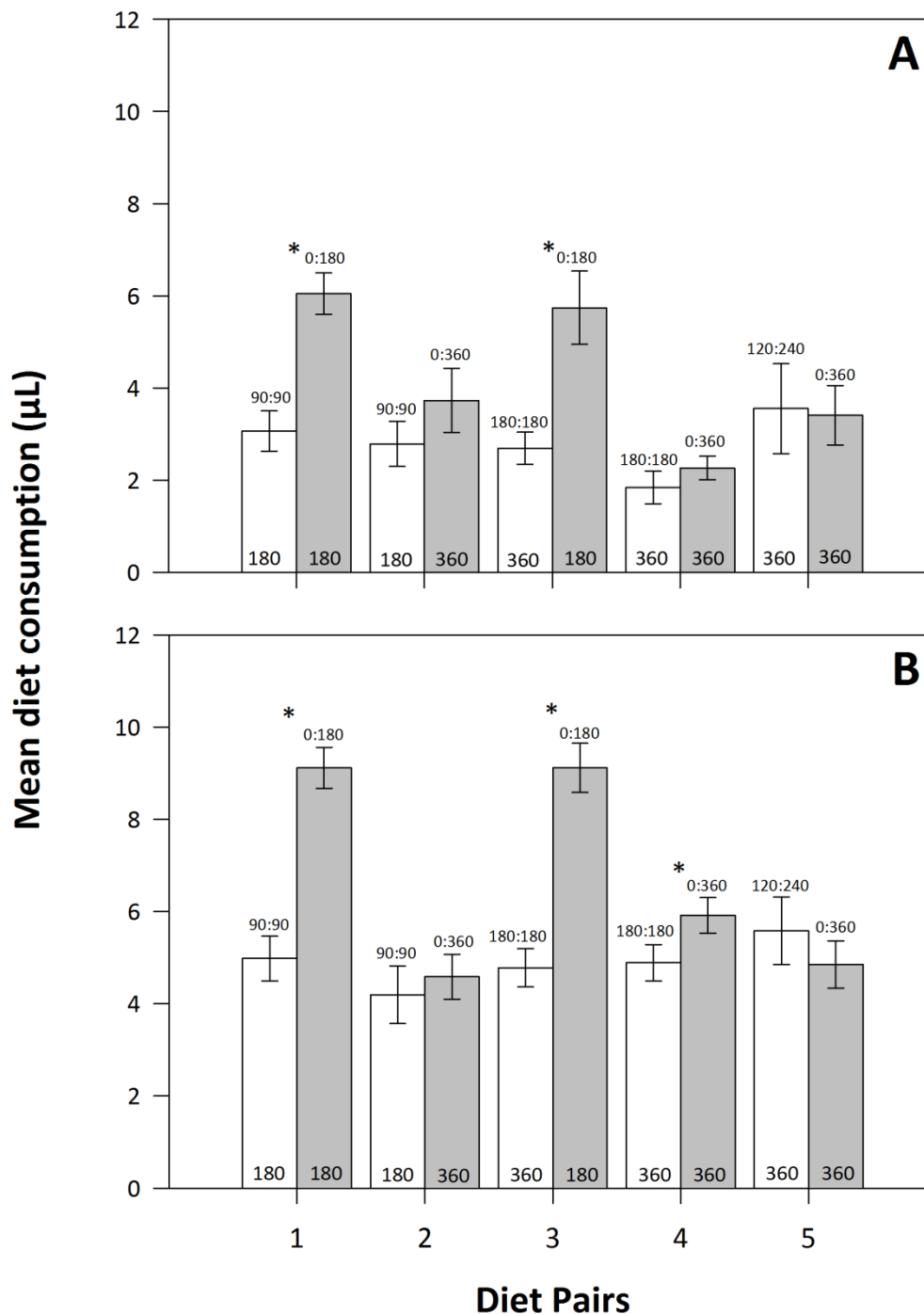


Figure S2: Mean (\pm SE) intake of diets in each of the diet pairs for **A** males and **B** females. In each diet pair, the protein rich diet is represented by the white bars and the carbohydrate rich diet by the grey bars. The P:C ratio of diets are provided above each bar and the total nutrition of each diet is provided at the base of each bar. A two-factor ANOVA examining the effect of diet pair, sex and their interaction on the difference in intake between the protein and carbohydrate rich diets showed a significant difference across diet pairs ($F_{4,132} = 33.1$, $P = 0.001$) but not across the sexes ($F_{1,132} = 0.1$, $P = 0.75$) nor

was there a significant interaction between diet pair and sex ($F_{4,132} = 1.1$, $P = 0.34$). Paired t -tests were used to determine which diet pairs the sexes showed a significant preference for one diet over the other (denoted by an asterisks).

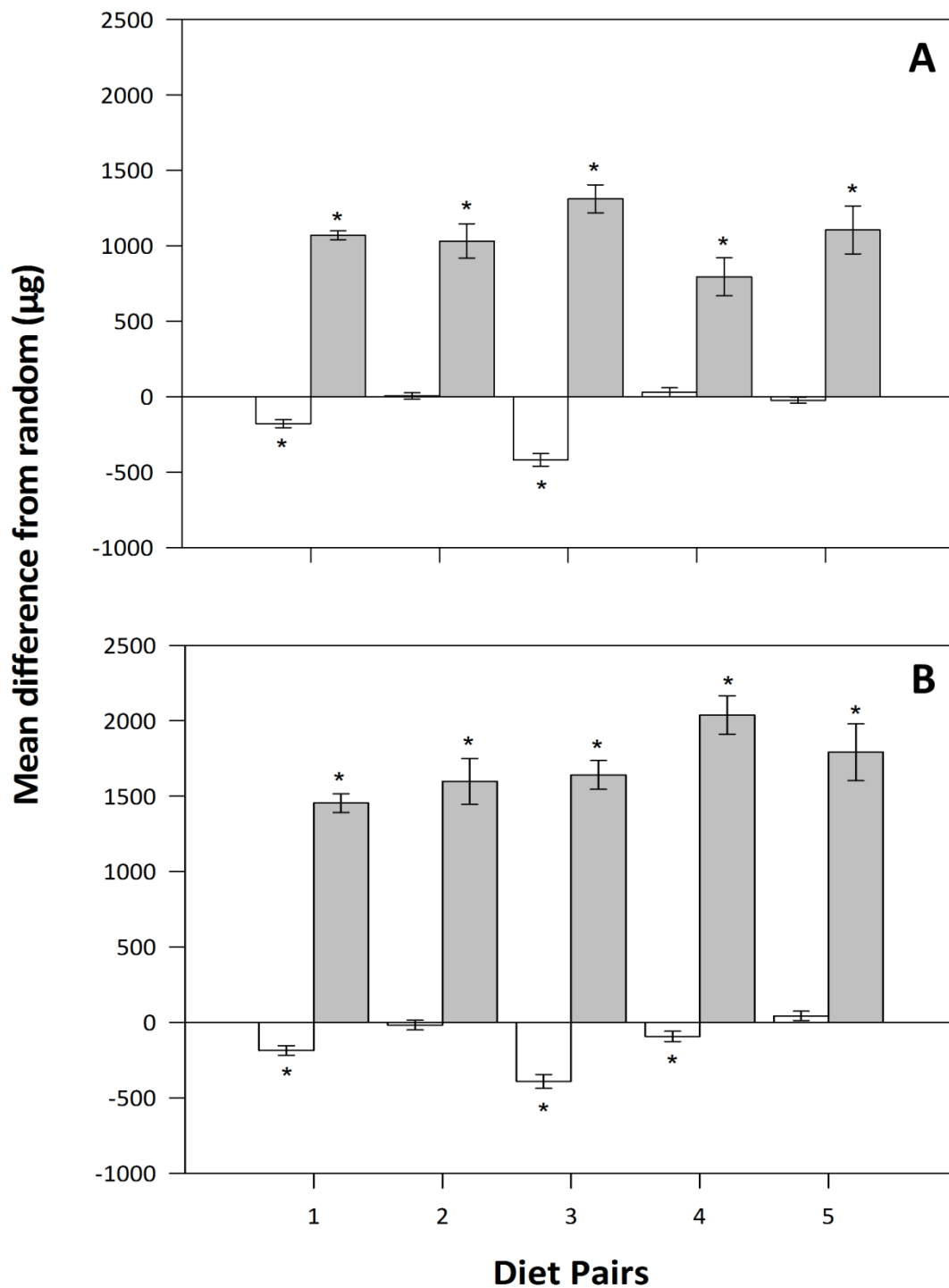


Figure S3: Mean (\pm SE) difference in the intake of protein (white bars) and carbohydrates (grey bars) for **A** males and **B** females from the random intake of these nutrients if flies

fed at random in the diet pair. One sample *t*-tests (testing against a mean of zero) were used to determine if nutrient intake was significantly greater or lower than random feeding (denoted by asterisks).

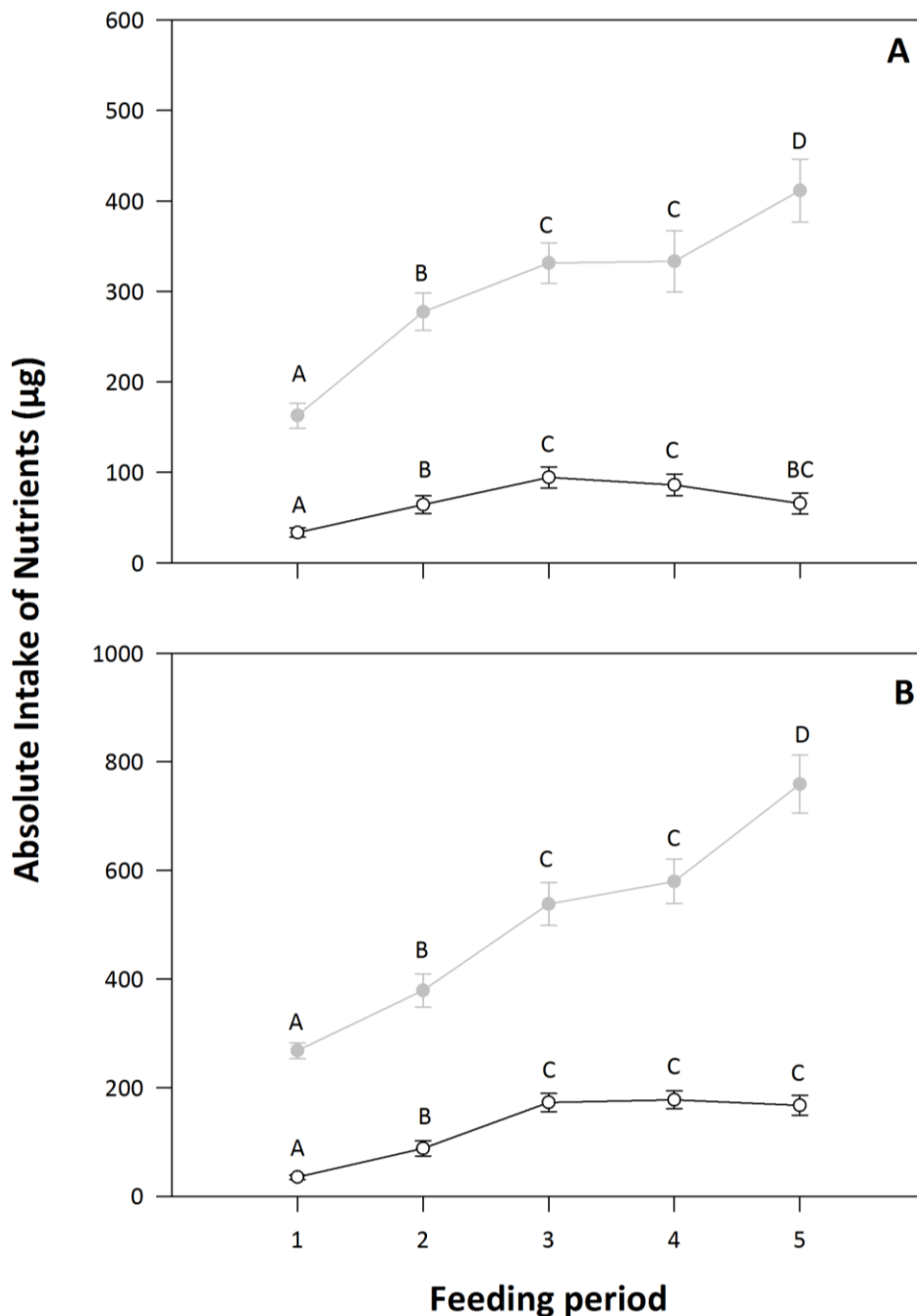


Figure S4: Mean absolute intake of protein (black, open symbols) and carbohydrates (grey, closed symbols) of **A** male and **B** female flies when given dietary choice over 5, 3-day feeding intervals.

Table S1: Nutrient contents and ratios of the 29 artificial liquid diets used in our experiments.

Diet	Protein (P) (g/L)	Carbohydrate (C) (g/L)	Concentration (P + C) (g/L)	Ratio (P:C) (g:g)
1	30.0	15.0	45.0	2:1
2	60.0	30.0	90.0	2:1
3	120	60.0	180	2:1
4	240	120	360	2:1
5	22.5	22.5	45.0	1:1
6	45.0	45.0	90.0	1:1
7	90.0	90.0	180	1:1
8	180	180	360	1:1
9	15.0	30.0	45.0	1:2
10	30.0	60.0	90.0	1:2
11	60.0	120	180	1:2
12	120	240	360	1:2
13	9.0	36.0	45.0	1:4
14	18.0	72.0	90.0	1:4
15	36.0	144	180	1:4
16	72.0	288	360	1:4
17	5.0	40.0	45.0	1:8
18	10.0	80.0	90.0	1:8
19	20.0	160	180	1:8
20	40.0	320	360	1:8
21	2.7	42.4	45.0	1:16
22	5.3	84.7	90.0	1:16
23	10.6	169	180	1:16
24	21.2	339	360	1:16
25	0.0	45.0	45.0	0:1
26	0.0	90.0	90.0	0:1
27	0.0	180	180	0:1
28	0.0	360	360	0:1
29	0.0	0.0	0.0	0:0

All liquid foods also contained cholesterol (4.0g/L), RNA from yeast (10.0 g/L), Vanderzant vitamin mixture (3.6 g/L), Wesson salt mixture (10.0 g/L), and methyl paraben (1.5 g/L).

Table S2: Amino acid mixture used to vary protein content in our artificial liquid diets.

Amino acid	g/(100g)
L-alanine	4.87
L-arginine	6.67
L-aspartic acid	7.05
L-cysteine	2.55
L-glutamic acid	24.5
Glycine	5.62
L-histidine	2.85
L-isoleucine	3.52
L-leucine	6.75
L-lysine	3.67
L-methionine	1.72
L-phenylalanine	4.42
L-proline	7.80
L-serine	4.87
L-threonine	3.37
L-tryptophan	1.80
L-tyrosine	3.00
L-valine	4.95

Table S3: Repeated Measures ANOVA examining the effect of sex, diet pair and time on the intake of protein and carbohydrates when provided with dietary choice. As significant differences were detected across diet pairs, we conducted post-hoc analysis comparing the intake of nutrients across the sexes within each of the five diet pairs and across all of the diet pairs. In the overall model, the lack of significance of the interaction terms between sex and time and between sex, diet pair and time indicate that the sexes share a common trajectory for the intake of protein and carbohydrates. The lack of significance of the interactions terms between sex and time in the post-hoc models further confirm this conclusion.

Model term	df	Protein		Carbohydrate	
		F	P	F	P
Sex (A)	1,132	29.6	0.001	66.5	0.001
Diet pair (B)	4,132	11.9	0.001	3.8	0.006
Time (C)	4,129	134	0.001	246	0.001
A x B	4,132	1.8	0.14	2.1	0.09
A x C	4,129	1.8	0.13	2.1	0.09
B x C	16,395	4.7	0.001	4.2	0.001
A x B x C	16,395	1.1	0.37	1.3	0.20
Post-hoc Analysis					
<i>Diet Pair 1</i>					
Sex (A)	1,32	6.0	0.02	3.7	0.001
Time (B)	4,29	32.1	0.001	250	0.001
A x B	4,29	2.0	0.13	2.0	0.12
<i>Diet Pair 2</i>					
Sex (A)	1,29	1.0	0.33	9.8	0.001
Time (B)	4,26	15.1	0.001	38.1	0.001
A x B	4,26	1.9	0.15	1.5	0.24
<i>Diet Pair 3</i>					
Sex (A)	1,25	12.0	0.002	19.4	0.001
Time (B)	4,22	56.2	0.001	123	0.001
A x B	4,22	2.3	0.09	2.1	0.12
<i>Diet Pair 4</i>					
Sex (A)	1,23	5.2	0.03	17.5	0.001
Time (B)	4,20	27.3	0.001	47.9	0.001
A x B	4,20	1.5	0.24	1.9	0.14
<i>Diet Pair 5</i>					
Sex (A)	1,23	6.0	0.02	7.4	0.01
Time (B)	4,20	14.6	0.001	20.6	0.001
A x B	4,20	1.8	0.18	1.4	0.28
<i>Across Diet Pairs</i>					
Sex (A)	1,140	23.6	0.001	57.9	0.001
Time (B)	4,137	89.5	0.001	204	0.001
A x B	4,137	1.8	0.14	1.9	0.11

Effects of diet, sex and infection on age-specific mortality of the fruit fly:

Diets which extend lifespan increase pathogen susceptibility

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Author Contributions: CDM and NKP devised the conceptual design of the experimental work. CDM produced the manuscript under the supervision of NKP. CDM conducted the majority of the experimental work. DPM conducted the pathogen load study. CDM conducted analysis of the data. JH provided diets and suggestions over experimental procedures.

3.1 Abstract

The sexes differ in how they regulate their immunity and how they age. Although studies have shown that the sexes vary in their response to nutrients, few have considered how diet may contribute to the sex-specific mortality patterns of infected and uninfected animals. Here, using isocaloric diets varying in protein:carbohydrate ratio, likelihood mortality trajectory fitting, and *in vivo* pathogen load techniques, we investigate diet-induced age-specific effects on ageing and infection in male and female *Drosophila melanogaster*. A trade-off between lifespan and infection resistance with diet was identified. For both sexes, individuals fed a lower protein:carbohydrate ratio diet lived longer, but were more susceptible to fungal infection than those fed a higher ratio diet. Age-specific analysis revealed that carbohydrate-rich diets reduced initial mortality and simultaneously increased ageing rates for both sexes, as well as conferring more latent but substantially greater effects of fungal infection on mortality. Pathogen load and diet had differing impacts between the sexes. Females had higher pathogen loads than males irrespective of diet. Infected males showed higher survival than infected females when fed a carbohydrate-rich diet (i.e. diets selected by infected and uninfected flies), but the relationship was reversed under untreated conditions. Thus females are more susceptible to the influence of fungal infection than males. In light of prior studies, our findings imply that females are protein-limited both through a higher selection pressure for reproductive investment and resisting sexually-contracted infections. Our results provide insights into how the sexes utilise protein and carbohydrates in the interplay between lifespan and immunity.

Running Title: Effects of diet and sex on pathogen susceptibility

Keywords: Age-specific mortality, immunonutrition, ageing, Gompertz, pathogen load, *Drosophila melanogaster*, *Metarhizium robertsii*

3.2 Introduction

It is known that the sexes vary in their optimal life history strategies due to anisogamy (Bateman 1948; Restif and Amos 2010) however the roles of diet and immunity in sexual dimorphism are less appreciated. Diet and immunity are implicit to the concept of anisogamy. While females experience a stronger selection pressure to survive and produce young (Trivers 1972; Carey et al. 1995), males are fitness-limited by their ability to access mate and therefore adopt a 'live fast, die young' strategy, maximising reproductive effort in place of survival and immunity (Rolff 2002; Bonduriansky et al. 2008; Maklakov et al. 2009; Zuk and Stoehr 2010). In line with this, males are often observed to have reduced immune function, resistance, and increased infection risk in comparison to females (Zuk and Stoehr 2002; Nunn et al. 2009; Fanson et al. 2013). The sexes clearly differ in their investments towards somatic cell maintenance and immune function, but can they utilise diet differently to meet the nutritional needs of these selected traits?

Nutrition has important impacts on life history parameters; impacts which differ between the sexes. Each sex has been shown to require a unique dietary composition to optimise their lifetime reproductive success (LRS) and immune performance (Maklakov et al. 2008; Fanson et al. 2013; Chapter 2). While carbohydrate-rich diets lead to maximal lifespan, protein-rich diets promote immune function and reduce susceptibility to infection, the magnitude of these effects is sex-dependent (Dobson and Bawden 1974; Fernandes et al. 1976; Readshaw and Gerwan 1983; Chandra 1993 and 1997; Tatar and Carey 1995; Vass and Nappi 1998; Lee et al. 2008; Fanson et al. 2009; Srygley et al. 2009; Rauw 2012). Clearly understanding how the sexes utilise available nutrients for ageing and immunity will shed light on the limitations of these traits.

The contribution of nutrient acquisition and utilisation to the trait differences between the sexes although identified, is largely unknown. Previous nutritional studies have been limited in their ability to control calorie and micronutrient concentration, aspects which influence fitness-related traits (Weindruch et al. 1986; Bauerfeind and Fischer 2005; Min and Tatar 2006). Recent studies accounting for these problems have found that while females require a diet richer in protein than males to optimise LRS, diets conferring maximal lifespan did not differ between the sexes (Maklakov et al. 2008; Chapter 2). Additionally, Fanson et al. (2013) observed that female flies had elevated

immune activation independent of diet and that immunity was more dependent on both protein and carbohydrate than in males. As diet choice between the sexes are similar, these results imply that the sexes differ in their utilisation of dietary components rather than their acquisition (Maklakov et al. 2008; Chapter 2). But how do the sexes utilise macronutrients such as protein and carbohydrate differently to produce optimal life strategies between immune and survival investments?

Survival is a common, indirect measure of fitness often reported as alterations in maximum or mean lifespan. Although identifying survival-altering treatments have assisted in our understanding of how ageing occurs and evolved (Guarente and Picard 2005), determining changes in age-specific characteristics of mortality informs us whether conditions reduce ageing rates or purely influence age-independent mortality risks (Pletcher 1999; Bronikowski & Flatt 2010). Furthermore age-specific effects of treatments such as diet or infection determines whether these effects inflict permanent or temporal life history adjustments within the host. Recent studies have been informative of the effects of diet on age-specific parameters of survival in female flies (Lee et al. 2008); however the differences in sexes have yet to be assessed.

Here we address how the sexes vary in age-dependent mortality rates and pathogen susceptibility as well as the role of nutrition in this variation. Using the fruit fly, *Drosophila melanogaster* as host for the insect-generalist entomopathogenic fungus, *Metarhizium robertsii*, we condition flies to a number of isocaloric holidic diets which vary in protein:carbohydrate ratio to determine the effects of these macronutrients on their life history. The fruit fly is well-suited for large-scale experimental demography, can be infected *en masse* with a natural mode of infection, and is amenable to *in vivo* pathogen load assessment (Kohler 1994; Priest et al. 2002; Mulcahy et al 2011; SII). Furthermore, our controlled diets remove noise from calorie and micronutrients fluctuations between diets (Min and Tatar 2006; Grandison et al. 2009; Fanson and Taylor 2012). With these systems we first investigate the effects of diet on the sex-specific survival of both uninfected and infected flies as well as their age-specific mortality. Following this, we assess *in vivo* pathogen growth in flies conditioned to specific diets which lie close to those chosen by uninfected and infected flies. Using these select diets we identify variation in pathogen load and susceptibility between the sexes.

3.3 Materials & Methods

Fly & Fungal Maintenance

A wild-type *Dahomey* strain of *Drosophila melanogaster* (obtained from Stuart Wigby, University of Oxford) was cultured in large population cages (1m³) with overlapping generations for two years prior to the start of the experiments. All experimental animals were maintained at 25°C with 12:12 light-dark cycle in standard *Drosophila* vials at low densities (approximately 50 flies per vial) for at least two generations prior to the start of experiments. Previous generations were bred on an oatmeal-molasses-agar media with added live baker's yeast and an antifungal agent (Nipagin), which inhibited the growth of naturally-occurring saprophytic fungi. All experimental flies used were collected over a period of 24 hours and held in vials at a density of 50 flies per vial. Once collected, flies were matured for 48 hours on standard food before being treated and placed into individual vials with artificial diets.

Metarhizium robertsii (isolate 2575) was obtained from the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF, United States Department of Agriculture). We inoculated ¼-strength sabouraud dextrose agar (SDA) with *M. robertsii* conidia (asexual fungal spores) and incubated the plates at 28°C for four weeks before storing at 4°C for up to three months. Conidia were collected by scraping the surface of the sporulating culture with an inoculating loop. Conidia were autoclaved by placing live spores into a glass universal which was enclosed within an autoclave bag. This ensured no moisture came into contact with the spores.

Diet Preparation

Diets followed a single isocaloric rail (360g/L) as suggested in the nutritional geometry approach of diet manipulation used in Fanson and Taylor (2012) and ranged in protein:carbohydrate (P:C) ratios, 2:1, 1:1, 1:2, 1:4 and 1:8. Protein consisted of 18 different amino acids (Chapter 2; Table S2) whereas carbohydrate consisted of sucrose. All diets also contained a fixed amount of cholesterol (4.00g/L), RNA from yeast (10.00g/L), Vanderzant vitamin mixture (3.60g/L), Wesson salt mixture (10.00g/L), and methyl paraben (1.50g/L). Our artificial diets therefore differed from those used by Fanson and Taylor (2012) where micronutrients were provided in direct proportion to the amount of protein contained in the diets. Once the correct mixture of P:C ratio and

micronutrients were measured, diet preparations of 2ml per vial were solidified with the addition of 1% agar in standard narrow *Drosophila* vials.

Fungal Exposure

At adult age day 3, approximately 300 mixed sex flies were treated with 20mg of live (infected) or autoclaved (pathogen challenged) conidia without CO₂ anaesthesia by agitating in a 250ml conical flask for 10 seconds. Exposed flies were held in temporary holding vials before being transferred to new food vials and finally transferred into appropriate vessels containing artificial diets. Sham-treated control flies were manipulated identically in an empty flask.

Demographic and pathogen load assay of diet, infection & pathogen challenge

Following treatment, flies were placed into 10 x 15cm demography cages (see Priest et al. 2002) of approximately 300 flies per cage with two replicate cages for each diet and treatment. Diets were replaced every two days and the number and sex of dead individuals were recorded daily until all flies in the experiment died.

Pathogen load was assessed on select P:C diets (1:2, 1:4 and 1:8) close to those chosen by uninfected and infected individuals (1:5.81 & 1:4.57 respectively; Chapter 4), and two treatments (untreated and live fungal treated). Flies were collected and treated as previously stated and allocated to particular diets. 96 hours following infection, 15 flies of each sex per treatment were removed, surface sterilised in bleach (1%), ethanol (70%) and sterile H₂O, before being homogenised and spread across a ¼-SDA plate. Plates were incubated for 96 hours at 25°C before the number of *Metarhizium*-like colony forming units (CFUs) were recorded (see SII).

Statistical analysis

All statistical analyses were performed with R version 2.15 (R Core Team 2012) and conformed to the assumptions of the statistical models.

First, we tested for a relationship between survival (i.e. the fold-increase in risk of death experienced by flies prior to the following interval), sex, treatment (naïve, pathogen challenged, or infected) and diet (P:C 2:1 – 1:8) using Cox proportional hazard models. In the full model, lifespan with the appropriate censoring information was the response variable with diet, treatment, sex and their pair-wise interactions as predictor

variables. Models with step-wise factor deletions were completed to assess the effects of individual factors and their interactions. Additionally, separate Cox regressions were performed for each diet and sex assessing the variation in survival between pathogen treatments and sham control. This measure accounted for the background responses of the organism's diet and sex. To visualise the effect of infection on survival for each diet, we calculated the difference in daily mortality values between infected and untreated (naïve) individuals, giving a measure of the diet and age-specific effects of fungal infection on survival for each sex.

Second, we tested for a difference in the risk of death between flies of different sex and diet allocation within treatments. In the full Cox models, age at death with appropriate censoring information was used as the response variable, and diet, sex and their interaction, as predictor variables for all treatments. Additionally, for each diet, the difference in the risk of death between the sexes was calculated and visualised. We included replicate number as a random effect for all Cox hazard proportion models; however, this was not a significant predictor.

Age-specific effects on survival of each diet, sex and treatment (excluding live infection) were investigated using maximum likelihood criteria for mortality model fitting of the Gompertz (Equation 1) using the *SURVOMATIC* package. The Gompertz model was used as model fits gave lower Akaike Information Criterion (AIC) values than did fittings to Exponential, Weibull, Gompertz-Makeham and Gompertz-Makeham logistic models.

$$\mu(t) = ae^{bt} \quad (1),$$

where $\mu(t)$ is mortality rate at age t , a is the mortality intercept, b is the rate of ageing and e is the exponential term (Pletcher 1999). Parameters of the model were fit to two-day mortality data averages for independent diets, treatments and sexes. Using *FINDPARS* function, differences between parameter values in the model fitting were compared between diets (comparing mortality values of the diet of interest with those experienced under a 1:8 P:C ratio), treatments and sexes independently.

Differences in pathogen load between sexes and diets (P:C 1:2 – 1:8) were assessed using linear models. In the full model, the response variable, CFU load, was tested with the predictor variables diet and sex. Separate models were used for each sex and for specific diet comparisons.

3.4 Results

Effects of sex, diet and treatment on survival and susceptibility

The full model showed that lifespan was significantly related to sex, diet and treatment (Table 1). For both sexes, diet was shown to affect survival across untreated, pathogen-challenged and infected individuals (Females: $\chi^2_4 = 490$, $P < 0.001$; $\chi^2_4 = 527$, $P < 0.001$; $\chi^2_4 = 648$, $P < 0.001$; Males: $\chi^2_4 = 696$, $P < 0.001$; $\chi^2_4 = 695$, $P < 0.001$; $\chi^2_4 = 972$, $P < 0.001$ respectively) although a general trend of increased lifespan was observed with lower P:C ratios across both sexes and all treatments (Figures 1a & b; Table 2). Diets which enhanced lifespan led to an increase in the risk of death, or pathogen susceptibility, of infected individuals for both sexes (Figures 1c & d).

Across all diets and treatments, survival differed between the sexes (Table 1), although within treatments, survival differed significantly for pathogen challenged and infected flies but not those untreated (Tables 2a, b & c). Pathogen challenge was not shown to affect lifespan in female individuals across all diets ($\chi^2_1 = 0.1$, $P = 0.743$; Figures 1a & c) while it increased lifespan in males across all diets except P:C 2:1 ($\chi^2_1 = 24.3$, $P < 0.001$; Figures 1b & d).

Effects of sex, diet and treatment on age-specific patterns of mortality

Generally it was seen, for both sexes under untreated and pathogen challenged conditions, that lower P:C ratios, although provided higher mean lifespans, resulted in higher ageing rates and lower initial mortalities (Figures 2a, b, c & d; Table 3). Consistent with evolutionary theory for both untreated and pathogen challenged flies, males were found to have higher rates of ageing and lower initial mortalities in comparison to females (Table 3). Ageing rates between the sexes did not differ at high P:C ratio diets although male age-independent mortality increased (Table 3). Furthermore for pathogen challenged flies, both males and females experienced increased age-dependent mortality and decreased age-independent mortality in comparison to untreated flies as seen previously with this treatment (Table S1; data not shown). This was observed at all diets except P:C 2:1. The additional effect of infection on diet-dependent and age-specific mortality was visualised for seven days post-infection (Figures 2e & f). Both sexes

experienced reduced and earlier effects of pathogen on mortality on protein-rich diets and later yet more substantial effects when fed more carbohydrate-rich diets.

Effects of sex and diet on the impact of infection

In vivo analysis across diets showed that pathogen load increased with carbohydrate in the diet (CFU count; $F_{2,60} = 101$, $P < 0.001$; Figure 3a). Sex differences were also found between the CFU counts ($F_{1,60} = 60.7$, $P < 0.001$): males had lower pathogen loads than females across all diets. Infected males fed carbohydrate-rich diets survived better (i.e. had a lower hazard ratio) than female flies (Figure 3b). When uninfected however, males experienced higher rates of mortality. Females therefore experience a greater impact of infection on mortality than males under carbohydrate-rich diets.

3.5 Discussion

The sexes differ in their life history traits including survival and immunity. Yet little is known of the role of nutrition in this variation, or how age-specific patterns in these traits differ between males and females. Here, we show that for both sexes, diets which confer maximal lifespan do so by reducing age-independent mortality and increasing ageing rates. Furthermore these diets confer both increased susceptibility to fungal infection and higher pathogen loads. Between the sexes, we find that females experience higher pathogen loads and susceptibility than males at diets preferred by infected individuals. These diets also provide higher survival in untreated females in relation to their male counterparts. This implies that females experience a greater impact of infection than males in protein-limited conditions. These results show the role diet plays in age-specific mortality patterns, how immune-competence varies and shed light on the importance of infection resistance in life history evolution between the sexes.

Although carbohydrate-rich diets promote longevity and protein-rich diets enhance immune properties, how nutrition influences the response to pathogen exposure and how these effects vary between the sexes are unclear (Maklakov et al. 2008 and 2009; Lee et al. 2008; Fanson and Taylor 2012). Here we find that the diet which enhances lifespan confers increased susceptibility to infection in both sexes revealing a cost to longevity-promoting conditions. Interestingly survival did not differ between the sexes under control conditions, however there was a clear difference in how the sexes responded to the exposure of heat-killed spores. While females experienced no significant change in their survival to the treatment across all diets, males lived longer under the majority of diets. Although this may seem an unusual result, previous studies have observed beneficial hormetic effects on survival from this treatment (see Chapter 5). That no such response was observed in females is consistent with previous work where males are found to be more sensitive to hormetic treatments than females (Sarup and Loeschcke 2011).

Across many species, including humans, it is known that females live longer than males (Promislow 2003; Bonduriansky et al. 2008). Although this is expected from higher selective pressures on female survival, age-specific patterns of mortality and the effects of diet have rarely been assessed (Trivers 1972; Clutton-Brock and Isvaran 2007). Age and diet-specific analysis of untreated and pathogen challenged flies revealed that females

age slower and have greater age-independent mortality in comparison to males almost independent of diet (i.e. P:C <2:1) as consistent with evolutionary theory. Furthermore, although carbohydrate-rich diets led to increased longevity for both sexes, age-independent mortality was reduced while ageing rate simultaneously increased for flies fed lower P:C diets. This result contradicts previous findings, which identify lower ageing rates in carbohydrate-rich diets in female flies (Lee et al. 2008). Our experiment, however, found similar patterns in both sexes and with larger sample sizes, echoing the results of a previous study flies (Zajitschek et al. 2014). Age-specific analysis also reveals that the effects of infection are reduced and experienced earlier in individuals fed protein-rich diets, a result consistent with previous findings that protein is important for immunity and pathogen resistance (Dobson and Bawden 1974; McClure 2009; Povey et al. 2009; Srygley et al. 2009; Cotter et al. 2011; Ponton et al. 2011). The use of more advanced modelling techniques on estimating the age-specific effects of pathogen on the host for individual diets may provide more conclusive insights into the influence of diet on pathogen resistance and susceptibility.

Sex differences in immune properties are well established and expected from evolutionary theory (McKean and Nunney 2005; Stoehr and Kokko 2006; Nun et al. 2009; Zuk and Stoehr 2010). Despite this, how immune variation translates to pathogen load and the impact of infection between the sexes has seldom been assessed (Lindsey and Altizer 2008). We found that pathogen load is higher in female than male flies independent of diet. Furthermore, we found an increase in the survival of infected males in comparison to females fed low P:C diets (i.e. < 1:2); diets close to those chosen by both uninfected and infected flies (P:C 1:5.8 and 1:4.6 respectively; Chapter 4). These results imply that females are protein-limited when fed low P:C ratio diets due to their reproductive demands which restrict their ability to divert protein to immune function. This contradicts the results of a recent study which found higher immune performance in females irrespective of diet (Fanson et al. 2013); therefore another explanation can be proposed. Females are known to have a higher probability of contracting fungal infections during copulation than males (see SII). Additionally, females are known to up-regulate specific genes anticipating mating events, genes which confer fungal resistance when contracting infection through copulation, but not when infected directly (Immonen and Ritchie 2012; SII). Taken together, these studies suggest that females experience a

stronger selection pressure than males to develop immune defences to resist sexually-contracted infections, defences which are likely protein-costly and provide no benefit to suppressing direct infections. Females would therefore have highly active immune properties while simultaneously experience higher fungal loads from direct infection in comparison to males.

Our results also provide insights into the role of diet in pathogen resistance (i.e. ability to control pathogen levels) and tolerance (i.e. ability to resist pathogen-induced damage) of the host (Ayres and Schneider 2008). Flies fed carbohydrate-rich diets experienced higher pathogen loads four days post infection while simultaneously living longer in relation to flies on protein-rich diets. It could be argued that flies fed low P:C ratio diets are investing in tolerance, limiting their ability to suppress internal pathogen growth (resulting in higher CFU loads) while enabling them to delay the effects of infection on their physiology (resulting in a longer life). This implies that pathogen resistance is protein-costly while tolerance requires carbohydrates. As infected animals are known to choose higher P:C ratio diets, this suggests that animals seek to increase their resistance rather than tolerance (Lee et al., 2006; Povey et al., 2009; Chapter 4) which is supported from our age-specific breakdown of infection on mortality between diets.

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3.6 Figures & Tables

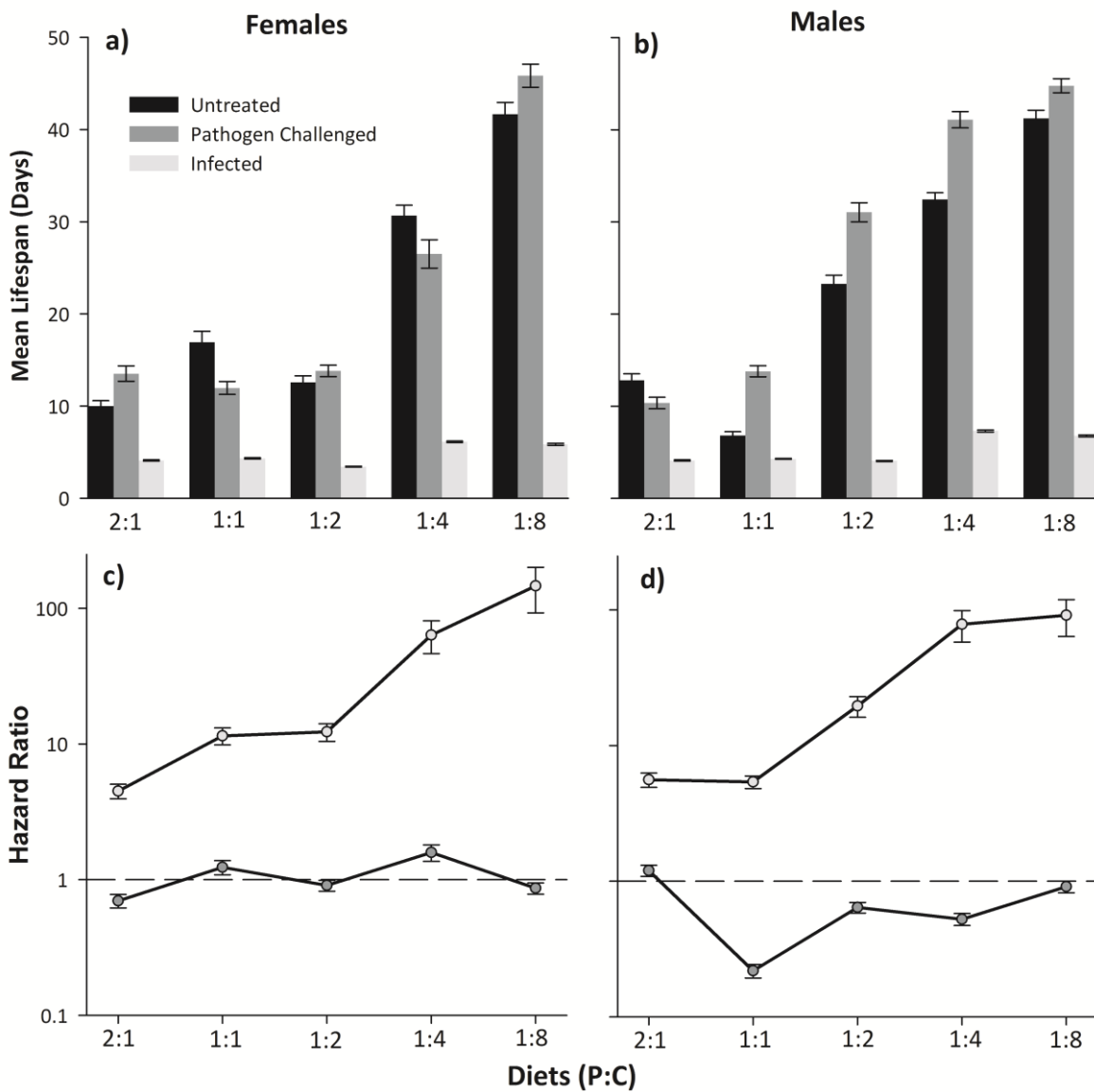


Figure 1: Mean lifespan of untreated, pathogen challenged and infected fruit flies across all diets for (a) females and (b) males (\pm SE). Untreated flies are shown in black, pathogen challenged in dark grey and infected flies in light grey. (c) & (d) represent survival hazard ratios experienced by infected (light grey) and pathogen challenged (darker grey) flies relative to untreated flies on each diet, for females and males respectively.

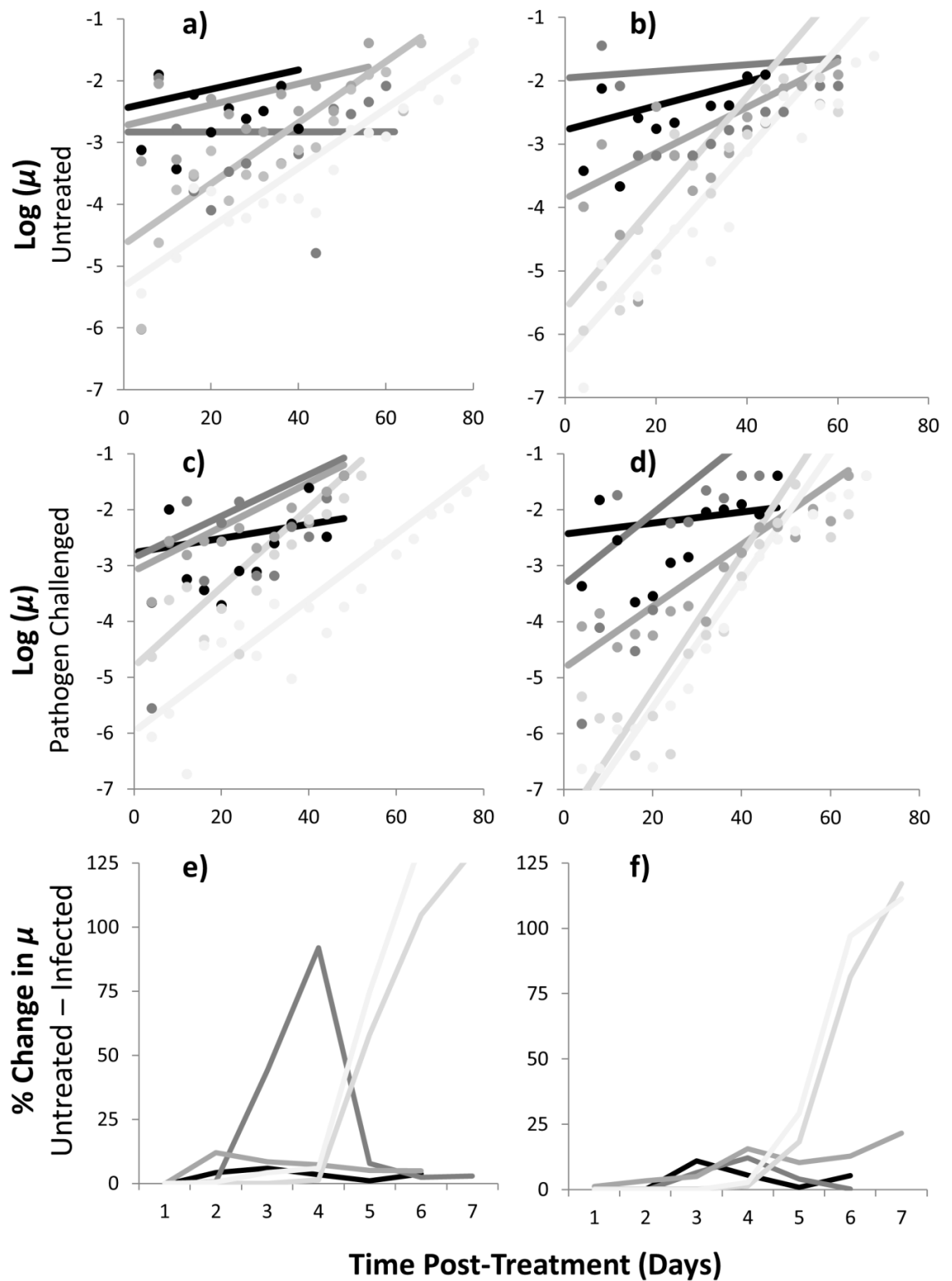


Figure 2: Age-specific effects of diet on female (a, c and e) and male (b, d and f) flies. High P:C ratio diets are shown in darker colours with a gradient towards the low P:C ratio diets in light grey. **a), b), c)** and **d)** show age-specific mortality fits to the Gompertz trajectory for untreated and pathogen challenged flies respectively. **e)** and **f)** represent age-specific

percentage changes in mortality between untreated and infected flies under various dietary conditions.

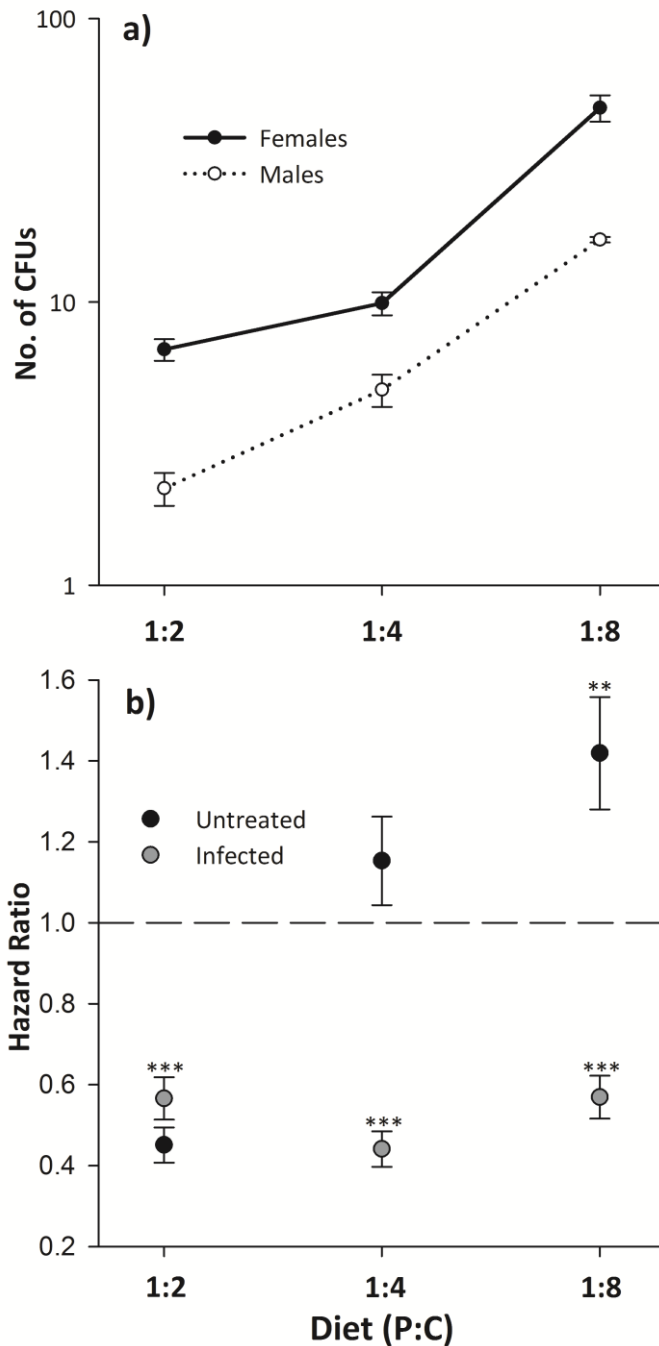


Figure 3: a) Effect of diet on sex-specific pathogen load in infected flies. Female data is shown by solid lines; male data is shown by dotted lines. **b)** Male risk of death in relation to female in uninfected (black) and fungal infected fruit flies (grey; \pm SE). Dashed line represents female survival. *** - $P < 0.001$, ** - $P < 0.01$

Table 1: Effects of sex, pathogen treatment, diet and their interactions on the survival of flies

Factor	DF	χ^2	P
Sex	1	4.96	0.026
Pathogen Treatment	2	4597	<0.001
Diet	4	1704	<0.001
Sex x Pathogen Treatment	2	59.5	<0.001
Diet x Sex	4	397	<0.001
Diet x Pathogen Treatment	8	323	<0.001
Sex x Diet x Pathogen Treatment	8	238	<0.001

Table 2: Effects of diet, sex and their interaction on Cox Hazard survival regressions of **a)** untreated, **b)** pathogen challenged and **c)** fungal infected flies.**a)**

Factor	DF	χ^2	P
Sex	1	2.8	0.092
Diet	4	962	<0.001
Diet x Sex	4	262	<0.001

b)

Factor	DF	χ^2	P
Sex	1	4.1	0.042
Diet	4	1085	<0.001
Diet x Sex	4	273	<0.001

c)

Factor	DF	χ^2	P
Sex	1	67.8	<0.001
Diet	4	1558	<0.001
Diet x Sex	4	72.8	<0.001

Table 3: Parameter values for Gompertz model fits to untreated female and male flies. *P* values indicate differences in the parameter values between the sexes.

Diet (P:C)	Parameter	Value		χ^2	<i>P</i>
		Female	Male		
2:1	<i>a</i>	0.087	0.062	2.6	0.023
2:1	<i>b</i>	0.016	0.019	0.1	0.717
1:1	<i>a</i>	0.059	0.141	34.6	<0.001
1:1	<i>b</i>	0.000	0.005	0.2	0.501
1:2	<i>a</i>	0.065	0.021	26.7	<0.001
1:2	<i>b</i>	0.017	0.036	3.4	0.009
1:4	<i>a</i>	0.010	0.004	8.9	<0.001
1:4	<i>b</i>	0.049	0.084	14.0	<0.001
1:8	<i>a</i>	0.005	0.002	7.3	<0.001
1:8	<i>b</i>	0.048	0.081	15.6	<0.001

3.7 Supplementary Figures & Tables

Table S1: Parameter values for Gompertz model fits to untreated (U) and pathogen challenged (T) male flies. *P* values indicate differences in the parameter values between the treatments.

Diet (P:C)	Parameter	Value		χ^2	<i>P</i>
		U	T		
2:1	<i>a</i>	0.062	0.086	2.9	0.015
2:1	<i>b</i>	0.019	0.010	0.5	0.316
1:1	<i>a</i>	0.141	0.035	44.6	<0.001
1:1	<i>b</i>	0.005	0.064	17.0	<0.001
1:2	<i>a</i>	0.021	0.008	13.3	<0.001
1:2	<i>b</i>	0.036	0.055	4.9	0.002
1:4	<i>a</i>	0.004	0.001	17.6	<0.001
1:4	<i>b</i>	0.084	0.121	8.9	<0.001
1:8	<i>a</i>	0.002	0.001	8.9	<0.001
1:8	<i>b</i>	0.081	0.114	8.8	<0.001

Evidence for adaptive modification of dietary preference under infection

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4.1 Abstract

Many animals eat less and alter the composition of their diet when they are infected. But the fitness benefits of these behavioural responses are unknown. Here we show that changes in the intake of protein and carbohydrates enhances resistance to infection and increases fitness. We found that the fruit fly, *Drosophila melanogaster*, is less susceptible to infection by the entomopathogenic fungus, *Metarhizium robertsii*, when it consumes a high protein and low calorie diet. In diet choice trials we found that infected flies choose to eat fewer total calories, thereby inducing anorexia, and a higher ratio of protein to carbohydrates. Interestingly, we find that healthy animals live longer and experience optimal fitness in carbohydrate-rich diets, while infected animals survive best and exhibit maximal life reproductive success in more protein-rich diets. These findings are important as they reveal the benefits of food choice in response to environmental stress, shedding light on how food sensing, choice and consumption evolves.

Running Title: Infection-induced dietary modification in the fruit fly

Keywords: Nutritional geometry, food preference, *Drosophila melanogaster*, *Metarhizium robertsii*, ecological immunity, calorie restriction, life history evolution.

4.2 Introduction

What animals eat is known to have a profound influence on their immunity and fitness, but as yet it is unclear if they use diet choice as a mechanism for fighting off pathogens and enhancing life reproductive success. Under favourable conditions animals choose diets with low protein to carbohydrate (P:C) ratios, which tend to maximise their fitness (Readshaw and Gerwan 1983; Tatar and Carey 1995; Fanson et al. 2009; Chapter 2). However, when infected animals appear to eat less and consume high P:C ratio diets (Bazar et al. 2005; Lee et al. 2006; Ayres and Schneider 2009; Povey et al. 2009). The benefits, if any, of this shift in the infected host's diet remains elusive.

Eating proportionately more protein and fewer total calories (often inappropriately termed anorexia, see Bulik et al. 2005 for a definition) could represent a potent mechanism for fighting off infections and enhancing fitness. Protein-rich diets result in higher immune function and a reduction in susceptibility to parasitism (Dobson and Bawden 1974; Fernandes et al. 1976; Chandra 1993 and 1997; Vass and Nappi 1998; Srygley et al. 2009). Furthermore, it has been suggested that a reduction in calorie intake influences resistance and tolerance to infection (Ayres and Schneider 2009; Povey et al. 2014).

Questions regarding the role of diet choice in adaptive responses to infection have been difficult to address due to methodological limitations. Many previous studies of infection have used diets which generate fluctuations in calorie and micronutrient composition (Min and Tatar 2006; Fanson and Taylor 2012) which can substantially influence life history (Weindruch et al. 1986; Bauerfeind and Fischer 2005; Grandison et al. 2009). This methodological limitation has generally been resolved through the advent of nutritional geometry (NG), which involves generating diets with varying concentrations and ratios of nutrients, accurate measurement of dietary intake, fitness assays and statistical modelling (Lee et al. 2008; Archer et al. 2009; see Chapter 2). Previous work, using NG, has found that animals change what and how much they eat when infected (Lee et al. 2006; Povey et al. 2009, 2014), but we don't know whether these behaviours are adaptive for infected hosts.

It is important to separate the costs of pathogen-induced stress and immune-stress on the host to interpret the true impact of infection. Although it is known that chronic immune stimulation is deleterious for the host, both in terms of their lifespan and

reproductive outputs, the nutritional constraints imposed from immune deployment have generally remained unstudied (Losdat et al. 2011; Pursall and Rolff 2011). Using a NG approach to investigate whether animals adjust their dietary composition, or whether they reallocate their available nutrients as some have suggested (Cotter et al. 2011), would provide insights into the maintenance and activation costs of the immune system.

Here we address these questions using the fruit fly, *Drosophila melanogaster* as host for the insect-generalist entomopathogenic fungus, *Metarhizium robertsii*. We use a NG approach to investigate the parameters of diet including calorie intake as well as protein and carbohydrate consumption on the life history of flies facing infection and immune deployment (see Archer et al. 2009). This system has several features that make it suitable for the study of fitness consequences of dietary modification: (1) Utilising NG and using basic amino acids as a source of protein avoids effects of calorie and micronutrient fluctuations on life history (Min and Tatar 2006; Grandison et al. 2009; Tatar 2011; Fanson and Taylor 2012); (2) The fruit fly is well-suited for large-scale experimental demography and can be infected *en masse* with a natural mode of infection (Kohler 1994; Priest et al. 2002; see SII); (3) The lifespan, daily reproductive effort and lifetime reproductive success of flies and the level of daily diet consumption can easily be assessed (Lee et al. 2008; Chapter 2); and, (4) as cuticular exposure of heat-killed fungal spores induces immune activation in insects, we can assess the nutritional costs of immune deployment in these animals (Xia et al 2001).

4.3 Materials & Methods

Fly and Fungal Maintenance

A wild-type Dahomey strain of *Drosophila melanogaster* (obtained from Stuart Wigby, University of Oxford) was cultured in large population cages (1m³) with overlapping generations for two years prior to the start of the experiments. All experimental animals were maintained at 25°C with 12:12 light-dark cycle in standard *Drosophila* vials at low densities (approximately 50 flies per vial) for at least two generations prior to the start of experiments. Previous generations were bred on an oatmeal-molasses-agar media with added live baker's yeast and an antifungal agent (Nipagin), which inhibited the growth of naturally-occurring saprophytic fungi. All experimental flies used were collected as virgins over a period of 24 hours and held in separate sex vials at a density of 50 flies per vial. Once collected, flies were matured for 48 hours on standard food before males were added to female vials in a 1:1 ratio. Allowing 24 hours to mate, females were then removed, treated and placed into individual vials with artificial diets with either a choice or no choice.

Metarhizium robertsii (isolate 2575) was obtained from the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF, United States Department of Agriculture). We inoculated ¼-strength sabouraud dextrose agar (SDA) with *M. robertsii* conidia (asexual fungal spores) and incubated the plates at 28°C for four weeks before storing at 4°C for up to three months. Conidia were collected by scraping the surface of the sporulating culture with an inoculating loop. Conidia were autoclaved by placing live spores into a glass universal which was enclosed within an autoclave bag. This ensured no moisture came into contact with the spores.

Diet Preparation

Diets followed the nutritional geometry model using a modified version as stated in Fanson and Taylor (2012). 29 artificial liquid diets were produced which varied in five levels of calorie concentration (0, 45, 90, 180 and 360g/L) and seven P:C ratios (2:1, 1:1, 1:2, 1:4, 1:8, 1:16, 0:1; Chapter 2 Table S1). Protein consisted of 18 different amino acids (Chapter 2 Table S2), whereas carbohydrate consisted of sucrose. All diets also contained a fixed amount of cholesterol (4.00g/L), RNA from yeast (10.00g/L), Vanderzant vitamin mixture (3.60g/L), Wesson salt mixture (10.00g/L), and methyl paraben (1.50g/L). Our

artificial diets therefore differed from those used by Fanson and Taylor (2012) where micronutrients were provided in direct proportion to the amount of protein contained in the diets. Our artificial diets were designed to cover the same nutrient space as the yeast and sucrose based diets used by Lee et al. (2008) and the artificial diets used by Fanson and Taylor (2012). We also included a single diet containing no protein or carbohydrate (Diet 29, Chapter 2 Table S1) to root our nutrition-fitness response surfaces at the origin. Liquid diets were provided in either a single (no choice) or a pair (choice) of 5 μ L microcapillary tubes (Drummond Microcaps; Ja et al. 2007; Lee et al. 2008) and the consumption of diets measured over 3-day feeding intervals. For each feeding period, the amount of diet in microcapillary tubes was measured to the nearest 0.25mm before and after each feeding period using a precision ruler (Lee et al 2008). To control for the evaporation of diets, two microcapillary tubes per diet were established in individual vials during each feeding period and diet loss measured as outlined above. Evaporation control vials were maintained in the same way as experimental vials with the exception that they did not contain a fly. Total diet consumption was estimated in each feeding period by subtracting the volume of diet in tubes before and after feeding and then subtracting the average loss of diet due to evaporation estimated from the two control tubes from this value. Total diet consumption was converted to an intake of specific nutrients by multiplying this volume by the nutrient composition of the diet provided in Chapter2 Table S1.

Fungal Exposure

At adult age day four, approximately 300 female flies were treated with 20mg of live or autoclaved (inactive) conidia without CO₂ anaesthesia by agitating in a 250ml conical flask for ten seconds. Exposed flies were held in temporary holding vials before being transferred to new food vials and finally transferred into appropriate vessels. Sham-treated control flies were manipulated identically in an empty flask.

Fitness assay in response to diet, infection and pathogen challenge

We assessed the effects of diet on survival and fecundity of female flies exposed to live (infected), inactive (pathogen challenged) or absent spores (untreated) on the full range of diets. Post-treatment, females were transferred to individual vials which contained

approximately 2ml of solid ringer-agar solution where flies were fed allocated diets. Every three days flies were moved to fresh ringer vials and re-fed. At this time, used microcapillary tubes were measured for the amount of food consumed. A cube (approximately 10mm³) of standard yeast-molasses food was added to used vials which were then held for 14 days at 25°C and subsequently frozen to count the number of hatched pupae for fecundity counts of each fly. Survival was checked daily to assess lifespan and food and fecundity measurements were taken when each fly died. Life reproductive success was therefore recorded for each individual fly.

Diet choice and fitness assay in response to infection and pathogen challenge

Using the same methods as stated above, survival and fecundity of flies with a diet choice were assessed. Each fly was given two microcapillary tubes of various P:C ratios and/or caloric dilution. One of five combinations were used which included 1:0 vs. 1:1 (360g/L), 1:0 (360g/L) vs. 1:1 (180g/L), 1:0 (180g/L) vs. 1:1 (360g/L), 1:0 vs. 1:1 (180g/L) and 1:0 vs. 2:1 (360g/L). The amount consumed for each tube was recorded every three days and on the day in which the fly died.

Statistical analysis

A multivariate response-surface approach (Lande and Arnold 1983) was used to estimate the linear and nonlinear (i.e. correlational and quadratic) effects of protein and carbohydrate intake on response variables (i.e. lifespan, daily and lifetime reproduction) for untreated, pathogen challenged and infected flies. Nutrition-fitness response surfaces were visualised using nonparametric thin-plate splines implemented in the *FIELDS* package of *R* version 2.13 (R Core Team 2012). We used a sequential model building approach to assess whether the linear and nonlinear effects of protein and carbohydrate intake differed for our response variables within and across the treatments (Draper and John 1988; South et al. 2011). When significant linear (i.e. protein and carbohydrate) or quadratic (i.e. P x P and C x C) effects were detected in these sequential models, univariate tests were used to determine which of the nutrients were responsible (South et al. 2011). As our response variables were measured in different units, they were standardised using a Z- transformation prior to analysis (South et al. 2011).

To determine if flies consumed significantly more of one diet in each pair, we compared the total absolute consumption of both diets using paired *t*-tests. To determine the implications of dietary choice for nutrient intake, we first calculated the expected intake of protein and carbohydrate for each fly assuming random diet consumption. This expected intake was then subtracted from the observed intake of these nutrients and the difference compared to a mean of 0 (i.e. expected of flies eating at random) using a one-sample *t*-test (South et al. 2011).

To assess whether the intake of nutrients changed with diet pair and treatment over the duration of the experiment, we analysed the cumulative intake of protein and carbohydrate using repeated-measures ANOVA. We included treatment, diet pair and time as main effects in this model, plus all possible interaction terms. Significant interactions between treatment and time and/or between treatment, diet pair and time would indicate that the flies from individual treatments follow different feeding trajectories over time. As this overall model showed significant differences in nutrient intake across diet pairs (Figures S1 & S2), we conducted post-hoc analysis within each of the pairs using a reduced model that included only treatment, time and their interaction. This same model was used to compare the average cumulative intake of nutrients across diet pairs.

The regulated intake point for each treatment, defined as the point in nutritional space to which animals regulate when provided with dietary choice (Simpson et al. 2004), was calculated as the mean total intake of protein and carbohydrate across all diet pairs. To determine if the regulated intake point differed between the treatments we used ANCOVA including treatment (main effect), total protein intake (covariate) and their interaction as model terms and total carbohydrate intake as the response variable. A significant treatment by protein intake interaction would indicate that flies of individual treatments have different regulated intake points.

4.4 Results

The fitness of infected flies is optimised at a high P:C ratio and low calorie diet

Infection alters how nutritional intake affects the life history of the fly. We find that diets with a higher P:C ratio and lower calorie content optimise the fitness of infected flies relative to their control counterparts (Figure 1). While untreated animals experienced optimal lifespan at 1:16/8 P:C ratio and a concentration of 180g/L, and optimal daily egg production and life reproductive success at 1:1 at 360g/L, infected fly's lifespan was optimised at 2:1 P:C ratio at approximately 140g/L while daily egg production and life reproductive success were maximised at 1:1 at a lower calorie concentration (approximately 100g/L; Figure 1; Table 2). The optimal diets differed significantly for all traits linearly between untreated and infected flies in both protein and carbohydrate. Additionally pathogen challenged animals, although followed a similar nutrition-fitness response surface to untreated flies, were significantly different from controls in all traits assessed, again in a linear fashion (Table 2). Within each of the treatments, lifespan was optimised at different nutritional optima than both daily egg production and life reproductive success, although life reproductive success and daily egg production did not (Tables 1 & 3). This suggests that nutritional trade-offs lie between traits to maximise fitness echoing previous findings (Chapter 2; Lee et al. 2008; Maklakov et al. 2008).

Flies self-medicate through diet choice

Flies chose to regulate their nutritional intake differently when infected (Figure 2). The five diet pairs for each treatment differed in their affects. An overall significant effect of treatment and diet pair was found, but their interaction was not significant suggesting that protein and carbohydrate affect fitness independently (Table 4). Both daily protein and carbohydrate intake contribute to the overall effect of treatment and diet pair. Post-hoc analyses revealed that this treatment effect was not due to differences between the pathogen challenge and untreated treatments, but rather between both the pathogen challenged (data not shown) and untreated treatments with those infected (Table 4).

Infected flies not only ate significantly less but also chose a more protein-biased diet (mean daily regulated intake points, P:C, untreated = 1:5.81, pathogen challenged = 1:5.15, infected = 1:4.57; Figure 2). Overall, there were significant differences in the regulated intake point across the 3 treatments ($F_{4,534} = 9.4$, $P < 0.001$) which was due to

the differences in both protein ($F_{2,267} = 4.3$, $P = 0.014$) and carbohydrate ($F_{2,267} = 18.5$, $P < 0.001$) intake. This effect was driven by infection as no significant difference was found in the regulated intake point between untreated and pathogen challenged flies ($F_{2,177} = 1.1$, $P = 0.339$). Untreated ($F_{2,177} = 16.5$, $P < 0.001$) and pathogen challenged ($F_{2,177} = 14$, $P < 0.001$) treatments versus those infected were found to be due to both protein and carbohydrate intake (data not shown).

4.5 Discussion

It is clear that pathogens alter the life history of their hosts (Hurd 2001; Bonds 2006), but how infection alters nutritional requirements and whether animals change their diet in order to fight off natural infections remains largely unknown. A number of animals ingest or harvest non-nutritive compounds during infection (Diamond 1999; Huffman 2001; Simone-Finstrom and Spivak 2012; Milan et al. 2012; Zhang et al. 2012). Furthermore some animals are observed to ingest more protein and eat fewer calories when parasitised (Lee et al. 2006; Adamo et al., 2007; McClure 2009; Povey et al. 2014; Bashir-Tanoli and Tinsley 2014), but we do not know whether these changes in dietary intake maximise fitness, and are therefore adaptive responses, or whether they are by-products of a hard-wired infection response. These questions are important to resolve because they have bearing on public health, ecoimmunology, and have fundamental implications for life history evolution (French 2009; Schulenburg et al. 2009). Here we show that dietary modification is an adaptive behaviour for fungal infected fruit flies.

The relationship identified between nutrition and life history in untreated animals is similar to other studies. In the no choice trial, we found that longevity is maximal for animals which consume large quantities of carbohydrates while fecundity is optimised at an even mixture of proteins and carbohydrates (0.40mg daily nutrient consumption at a P:C ratio of 1:1.3). We also found the diets chosen by untreated flies (0.27mg daily consumption at P:C ratio of 1:5.8) are distant in nutrient space from the diets which optimise longevity or fecundity. Both of these observations are similar to those found in caterpillars, crickets, Q- and fruit- flies (Lee et al. 2008; Maklakov et al. 2008; Cotter et al. 2011; Fanson and Taylor 2012).

Previous studies have argued that the mismatch between diet optimality and choice occurs because of evolutionary constraints; i.e. that the life history differences prevent the evolution of a diet choice which maximises fitness (Lee et al. 2008; Archer et al. 2009; Chapter 2). But there has been little consideration of the evolutionary constraints imposed by infection. We found a mismatch between diet choice and trait peak in infected animals with respect to fecundity, but not with survival. Lifespan of infected animals is optimal at diets of intermediate calorie concentration over a broad range of P:C ratio (from 2:1 – 1:4). We find that infected animals chose diets on the edge of this range (0.18mg at a P:C ratio of 1:4.6). Despite their diet choice, we found that the

fitness of infected flies was optimal at a daily intake of 0.21mg at a P:C ratio of 1:1. The difference of 1.2 in the P:C ratio of diets chosen by naïve and infected flies is similar to previous findings in bacterial infected worms (Povey et al. 2009) and larger than identified in virally infected caterpillars (Povey et al. 2014). Taken together, these results reveal that flies behaviourally alter their diet, ingesting proportionally more protein and fewer calories to increase their life reproductive success under infection.

Reducing the overall intake of calories increases pathogen tolerance while force-feeding increases the susceptibility of hosts (Murray and Murray 1979; Kyriazakis et al. 1998; Adamo et al. 2007; Ayres and Schneider 2009; Povey et al. 2014), but we do not know whether reducing intake influences host fitness and thus whether it's an adaptive response. We find that a reduction in the ingestion of calories when infected enhances host fitness and is therefore an adaptive behaviour. Comparing the choice and no choice studies reveals that reducing daily intake (from approximately 0.36mg for untreated animals to approximately 0.21mg for those infected) enhances the fecundity and life reproductive success of infected flies, which would otherwise be costly for naïve animals.

Although a reduction in dietary intake is commonly observed, how this behaviour benefits the host is not clear (Plata-Salamán 1996; Bashir-Tanoli and Tinsley 2014). While previous studies have focused on how calorie limitation affects host immunity or pathogen tolerance, many have disregarded the costs of immune deployment at various nutritional intakes (McKean et al. 2008; Cotter et al. 2011). Although a recent study suggests that the costs of immune deployment are due to a behavioural reduction in feeding (Bashir-Tanoli and Tinsley 2014), when assessing fitness our results propose that the associated costs are reduced at lower calorie intakes. In pathogen challenged flies, a treatment which stimulates immune function in insects (Xia et al. 2001; Chapter 5), maximal lifespan and life reproductive success are reduced (from 14 to 11 days and 70 to 55 offspring at optimal values respectively) relative to their naïve counterparts. Furthermore lifespan and fitness, although consistently respond linearly to macronutrient intake, diverged in their response to carbohydrate, and to carbohydrate & protein respectively between treatments. This indicates that with increasing nutrient concentration the differences in the response to nutrients become greater, increasing the costs of immune deployment for the host.

The field of ecoimmunology has highlighted the importance of understanding how immunity trades-off with other fitness-related traits in order to assess the true costs of immune deployment and maintenance (Lochmiller and Deerenberg 2000; Zuk and Stoehr 2002; Siva-jothy et al. 2005; French et al. 2009; Flatt 2011; Hawley and Altizer 2011; Rauw 2012). Previous studies have suggested that immune activation incurs protein costs to the organism which, in response, redistributes nutrients rather than increasing their intake (Bonneaud et al. 2003; Cotter et al. 2011). However the nutritional costs on fitness-related traits are unknown. In concordance, we find that immune-elicited animals do not significantly alter their dietary intake but instead redistribute their available nutrients. Pathogen challenged flies, although show reduced lifespan, experience relatively little change in daily egg production (optimal values from 5.5 to 6) indicating that the costs of immunity are predominantly present in the survival of the organism. As lifespan is predominantly determined through carbohydrate intake, it is apparent that challenged flies are redirecting carbohydrates from survival to immunity while maintaining reproductive investment, rather than adjusting their overall intake. Our results assist in understanding how animals respond to immune deployment, and how the immune system has evolved within the context of resource-demanding traits (Rauw 2012). We show that the costs of immune activation cannot be recovered through diet and primarily affect survival rather than fecundity.

In conclusion, this is the first study to show that (a) fruit flies modify their diet to enhance fitness when infected; (b) reducing calorie intake when infected is an adaptive behaviour; and (c) confirms immune deployment confers nutritional-constraints to survival. We highlight the importance of immunological studies in considering how animals manipulate their nutritional environment when infected as well as the importance of protein intake in developing potential treatments in humans (Chandra 1999; Catalán et al. 2011). Overall our study suggests that dietary manipulation is an important method for animals to alleviate, to an extent, the fitness costs of infection.

4.6 References

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4.7 Tables & Figures

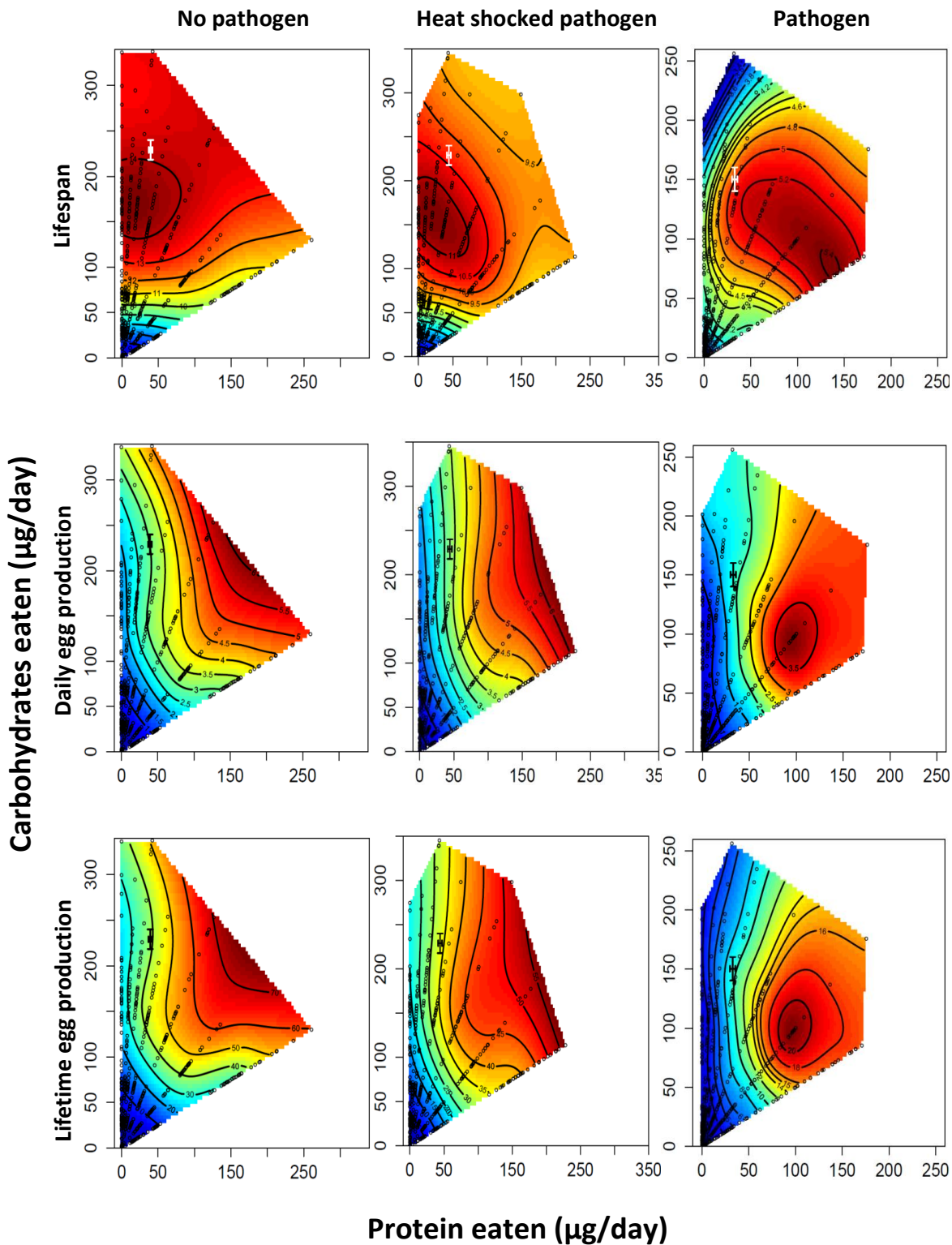


Figure 1: Nonparametric thin-plate spline contour visualisations of the responses surfaces describing the effects of protein and carbohydrate intake on lifespan, daily egg production and life reproductive success in *Drosophila melanogaster* treated with sham control (no pathogen), pathogen challenge (heat shock pathogen) and live infection (pathogen). Individual flies were allowed to feed *ad libitum* from one of the 29 liquid

foods across their adult lifespan. Open black circles represent the intake of protein and carbohydrate along each of the 7 nutritional rails by individual flies. The regulated intake point (\pm SE) for flies given the choice between 2 nutritionally complementary diets is mapped in the nutrient space (in white or black).

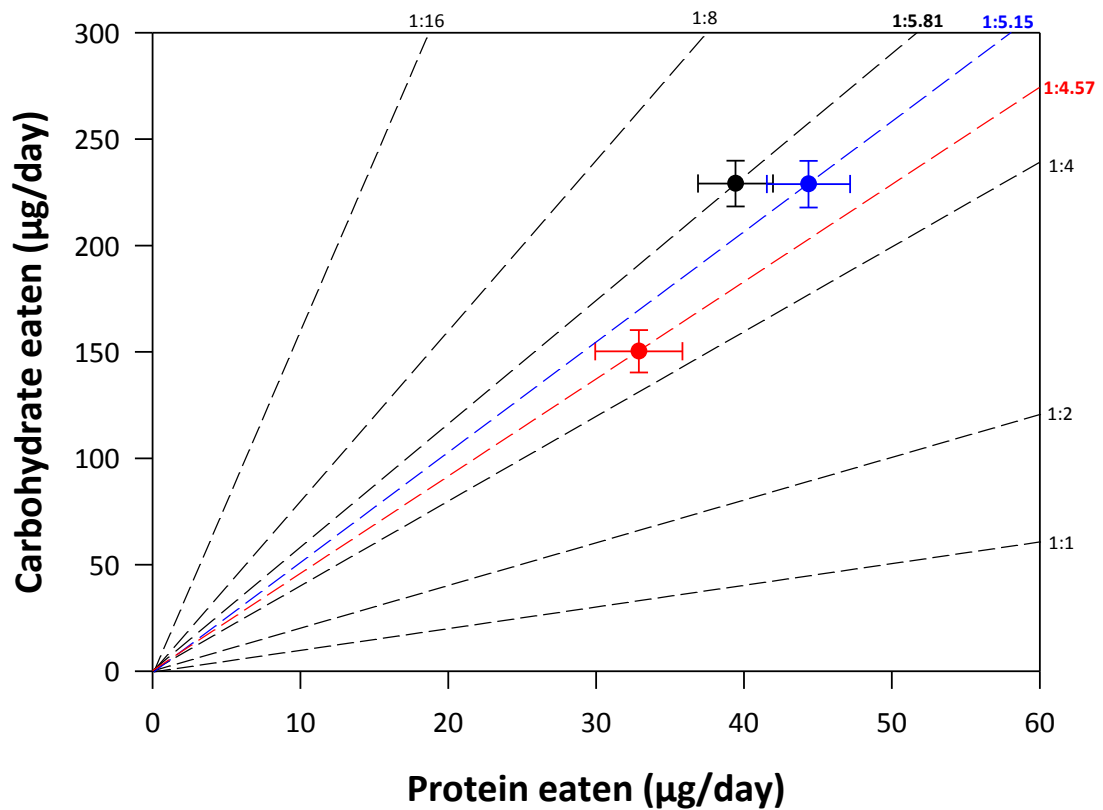


Figure 2: Daily average cumulative regulated intake points of protein and carbohydrate (mean \pm SE) for sham treated (black), pathogen challenged (blue) and infected (red) *Drosophila melanogaster* calculated over the first 15 days of adulthood when given the choice across all diet pairs.

Table 1: Sequential *F*-tests comparing the differential effects of protein and carbohydrate intake on lifespan, daily egg production and life reproductive success in infected, pathogen challenged and untreated female flies.

	<i>SS_R</i>	<i>SS_C</i>	<i>DF₁</i>	<i>DF₂</i>	<i>F</i>	<i>P</i>
Untreated						
<i>Lifespan vs. Daily egg production</i>						
Linear	627	580	2	1036	41.3	0.001 ^A
Quadratic	507	468	2	1032	42.6	0.001 ^B
Correlational	466	461	1	1030	10.2	0.001
<i>Lifespan vs. Life reproductive success</i>						
Linear	608	579	2	1036	26.2	0.001 ^C
Quadratic	482	457	2	1032	29.1	0.001 ^D
Correlational	452	444	1	1030	8.6	0.001
<i>Daily egg production vs. Life reproductive success</i>						
Linear	550	547	2	1036	2.2	0.11
Quadratic	515	513	2	1032	1.6	0.20
Correlational	494	494	1	1030	0.7	0.40
Pathogen challenged						
<i>Lifespan vs. Daily egg production</i>						
Linear	716	659	2	1036	45.1	0.001 ^E
Quadratic	578	551	2	1032	25.3	0.001 ^F
Correlational	551	542	1	1030	17.2	0.001
<i>Lifespan vs. Life reproductive success</i>						
Linear	720	678	2	1036	31.7	0.001 ^G
Quadratic	571	556	2	1032	14.8	0.001 ^H
Correlational	555	550	1	1030	10.9	0.001
<i>Daily egg production vs. Life reproductive success</i>						
Linear	559	557	2	1036	1.4	0.24
Quadratic	522	520	2	1032	1.7	0.18
Correlational	513	512	1	1030	0.7	0.40
Infected						
<i>Lifespan vs. Daily egg production</i>						
Linear	813	805	2	1020	4.9	0.008 ^I
Quadratic	765	751	2	1016	9.8	0.001 ^J
Correlational	749	749	1	1014	0.1	0.82
<i>Lifespan vs. Life reproductive success</i>						
Linear	789	776	2	1020	8.7	0.001 ^K
Quadratic	725	717	2	1016	5.7	0.003 ^L
Correlational	713	712	1	1014	0.4	0.54
<i>Daily egg production vs. Life reproductive success</i>						
Linear	721	720	2	1020	0.6	0.58
Quadratic	701	700	2	1016	0.7	0.51
Correlational	697	697	1	1014	0.7	0.40

Univariate tests: ^A = $P: F_{1,1036} = 76.15, P = 0.0001$; ^C = $P: F_{1,1036} = 17.33, P = 0.0001$; ^B = $P: F_{1,1032} = 9.04, P = 0.003$, ^D = $P: F_{1,1032} = 78.56, P = 0.0001$; ^E = $P: F_{1,1036} = 50.82, P = 0.0001$; ^F = $P: F_{1,1036} = 6.82, P = 0.009$; ^G = $P: F_{1,1032} = 10.27$,

$P = 0.001$, C: $F_{1,1032} = 50.10$, $P = 0.0001$; ^D = P: $F_{1,1036} = 89.52$, $P = 0.0001$, C: $F_{1,1036} = 9.24$, $P = 0.002$; ^F = P: $F_{1,1032} = 1.56$, $P = 0.21$, C: $F_{1,1032} = 49.16$, $P = 0.0001$; ^G = P: $F_{1,1036} = 63.28$, $P = 0.003$, C: $F_{1,1036} = 4.08$, $P = 0.04$; ^H = P: $F_{1,1032} = 2.54$, $P = 0.11$, C: $F_{1,1032} = 27.22$, $P = 0.0001$; ^I = P: $F_{1,1020} = 9.73$, $P = 0.002$, C: $F_{1,1020} = 1.74$, $P = 0.19$; ^J = P: $F_{1,1016} = 0.00$, $P = 0.99$, C: $F_{1,1016} = 19.53$, $P = 0.0001$; ^K = P: $F_{1,1020} = 17.30$, $P = 0.0001$, C: $F_{1,1020} = 2.18$, $P = 0.14$; ^L = P: $F_{1,1016} = 0.01$, $P = 0.93$, C: $F_{1,1016} = 11.46$, $P = 0.001$;

Table 2: Sequential F -tests comparing the effects of protein and carbohydrate intake on lifespan, daily egg production and life reproductive success between flies on different treatments. The sequential F -tests assess the differences in the sign and strength of the linear, quadratic and correlational regression gradients across different response variables between the treatments. When significant differences in linear or quadratic regression gradients were detected, univariate tests were used to determine whether this overall effect was due to the intake of protein or carbohydrate (or both).

	SS_R	SS_C	DF_1	DF_2	F	P
Untreated vs. pathogen challenged						
<i>Lifespan</i>						
Linear	702	696	2	1036	4.4	0.01 ^A
Quadratic	499	499	2	1032	0.4	0.70
Correlational	497	495	1	1030	2.8	0.10
<i>Daily egg production</i>						
Linear	546	542	2	1036	4.1	0.02 ^B
Quadratic	519	518	2	1032	0.5	0.59
Correlational	506	506	1	1030	0.1	0.77
<i>Life productive success</i>						
Linear	567	561	2	1036	5.1	0.006 ^C
Quadratic	513	513	2	1032	0.5	0.59
Correlational	501	499	1	1030	3.8	0.05
Untreated vs. infected						
<i>Lifespan</i>						
Linear	782	737	2	1028	31.6	0.001 ^D
Quadratic	595	589	2	1024	4.8	0.009 ^E
Correlational	589	588	1	1022	1.8	0.19
<i>Daily egg production</i>						
Linear	671	649	2	1028	18.1	0.001 ^F
Quadratic	630	629	2	1024	0.3	0.73
Correlational	624	622	1	1022	2.9	0.09
<i>Life reproductive success</i>						
Linear	653	619	2	1028	28.8	0.001 ^G
Quadratic	584	584	2	1024	0.5	0.62
Correlational	570	569	1	1022	2.7	0.10

Univariate tests: ^A = $P: F_{1,1036} = 0.13, P = 0.71$, $C: F_{1,1036} = 8.67, P = 0.003$; ^B = $P: F_{1,1036} = 3.72, P = 0.054$, $C: F_{1,1036} = 6.06, P = 0.014$; ^C = $P: F_{1,1036} = 3.96, P = 0.047$, $C: F_{1,1036} = 8.26, P = 0.004$; ^D = $P: F_{1,1028} = 17.66, P = 0.0001$, $C: F_{1,1028} = 58.11, P = 0.0001$; ^E = $P: F_{1,1024} = 4.44, P = 0.035$, $C: F_{1,1024} = 5.66, P = 0.018$; ^F = $P: F_{1,1028} = 0.001, P = 0.98$, $C: F_{1,1028} = 33.67, P = 0.0001$; ^G = $P: F_{1,1028} = 7.12, P = 0.008$, $C: F_{1,1028} = 57.13, P = 0.0001$; ^H = $P: F_{1,1028} = 13.21, P = 0.0001$, $C: F_{1,1028} = 20.69, P = 0.0001$; ^I = $P: F_{1,1028} = 2.89, P = 0.09$, $C: F_{1,1028} = 12.99, P = 0.0001$; ^J = $P: F_{1,1028} = 0.64, P = 0.42$, $C: F_{1,1028} = 23.04, P = 0.0001$.

Table 3: Linear and nonlinear effects of protein (P) and carbohydrate (C) intake on lifespan and reproduction in female *Drosophila melanogaster* when infected, treated with this heat-killed pathogen (pathogen challenge) and untreated.

Response variable	Linear effects		Nonlinear effects		
	P	C	P x P	C x C	P x C
Untreated					
<i>Lifespan</i>					
Gradient ± SE	0.05 ± 0.03	0.63 ± 0.03	-0.01 ± 0.02	-0.35 ± 0.02	-0.03 ± 0.04
t_{516}	1.3	18.3	0.2	15.2	0.6
<i>P</i>	0.19	0.001	0.82	0.001	0.56
<i>Daily egg production</i>					
Gradient ± SE	0.46 ± 0.03	0.43 ± 0.03	-0.10 ± 0.02	-0.08 ± 0.03	0.18 ± 0.05
t_{516}	14.1	13.3	4.5	3.1	3.8
<i>P</i>	0.001	0.001	0.001	0.002	0.001
<i>Life reproductive success</i>					
Gradient ± SE	0.38 ± 0.03	0.51 ± 0.03	-0.11 ± 0.02	-0.15 ± 0.03	0.23 ± 0.05
t_{516}	11.8	15.6	5.0	6.0	5.1
<i>P</i>	0.001	0.001	0.001	0.001	0.001
Pathogen Challenged					
<i>Lifespan</i>					
Gradient ± SE	0.06 ± 0.04	0.48 ± 0.04	-0.03 ± 0.03	-0.32 ± 0.03	-0.13 ± 0.05
t_{516}	1.6	12.1	1.1	11.0	2.6
<i>P</i>	0.10	0.001	0.29	0.001	0.01
<i>Daily egg production</i>					
Gradient ± SE	0.55 ± 0.03	0.32 ± 0.03	-0.08 ± 0.04	-0.13 ± 0.03	0.16 ± 0.05
t_{516}	16.8	9.9	3.2	4.7	3.3
<i>P</i>	0.001	0.001	0.001	0.001	0.001

<i>Life reproductive success</i>					
Gradient ± SE	0.48 ± 0.03	0.37 ± 0.03	-0.10 ± 0.03	-0.18 ± 0.03	0.10 ± 0.05
t_{516}	14.1	11.0	3.6	6.5	2.1
P	0.001	0.001	0.001	0.001	0.04
Infected					
<i>Lifespan</i>					
Gradient ± SE	0.28 ± 0.04	0.21 ± 0.04	-0.08 ± 0.03	-0.27 ± 0.04	0.07 ± 0.06
t_{493}	6.4	4.9	3.1	7.0	1.2
P	0.001	0.001	0.002	0.001	0.22
<i>Daily egg production</i>					
Gradient ± SE	0.46 ± 0.04	0.13 ± 0.04	-0.08 ± 0.03	-0.05 ± 0.04	0.05 ± 0.06
t_{493}	11.4	3.3	3.0	1.3	0.9
P	0.001	0.001	0.003	0.20	0.35
<i>Life reproductive success</i>					
Gradient ± SE	0.52 ± 0.04	0.13 ± 0.04	-0.09 ± 0.03	-0.12 ± 0.04	0.11 ± 0.05
t_{493}	13.4	3.3	3.7	3.4	2.2
P	0.001	0.001	0.001	0.001	0.03

Table 4: MANOVA tests comparing the effects of treatment, diet pair and their interaction in the daily intake of protein and carbohydrate of female flies given a choice of diet. Comparisons between all treatments, untreated and pathogen challenged, and between untreated and infected treatments were completed. Univariate tests were used to determine whether this overall effect was due to the intake of proteins or carbohydrates (or both).

	<i>DF₁</i>	<i>DF₂</i>	<i>F</i>	<i>P</i>
<i>Overall</i>				
Treatment	4	510	10.6	0.001 ^A
Diet Pair	8	510	13.6	0.001 ^B
Interaction	16	510	0.74	0.759
<i>Untreated vs. pathogen challenged</i>				
Treatment	2	169	1.50	0.226
Diet Pair	8	340	11.1	0.001
Interaction	8	340	0.65	0.739
<i>Untreated vs. infected</i>				
Treatment	2	169	20.6	0.001
Diet Pair	8	340	9.90	0.001
Interaction	8	340	0.71	0.684

Univariate tests: ^A = P: $F_{1,255} = 4.86$, $P = 0.008$, C: $F_{1,255} = 20.28$, $P = 0.0001$; ^B = P: $F_{4,255} = 10.75$, $P = 0.0001$, C: $F_{4,255} = 20.28$, $P = 0.0001$.

4.8 Supplementary Figures & Tables

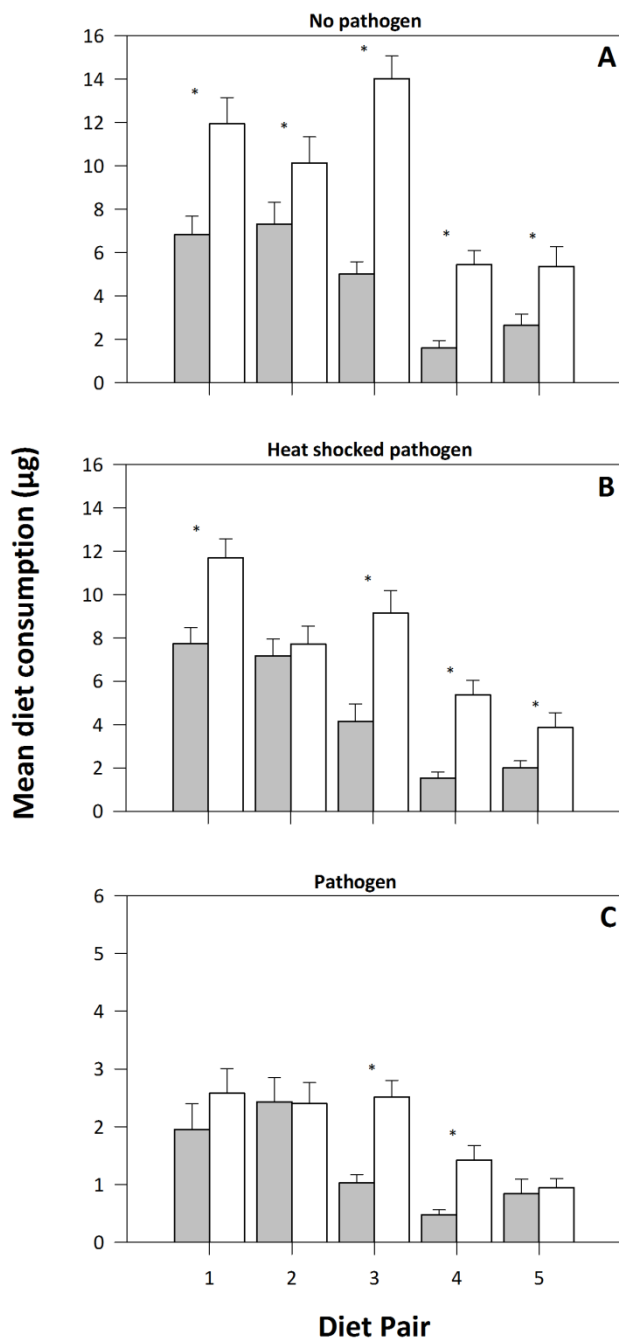


Figure S1: Overall consumption for flies on diet choice for each of the diet pairs (1-5; \pm SE) for **A** untreated flies, **B** pathogen challenged (heat shocked pathogen) flies, and **C** infected flies. In each diet pair, the protein rich diet is represented by the white bars and the carbohydrate rich diet by the grey bars. * indicates a significant difference ($P < 0.05$) of ingestion between the diets within the pair.

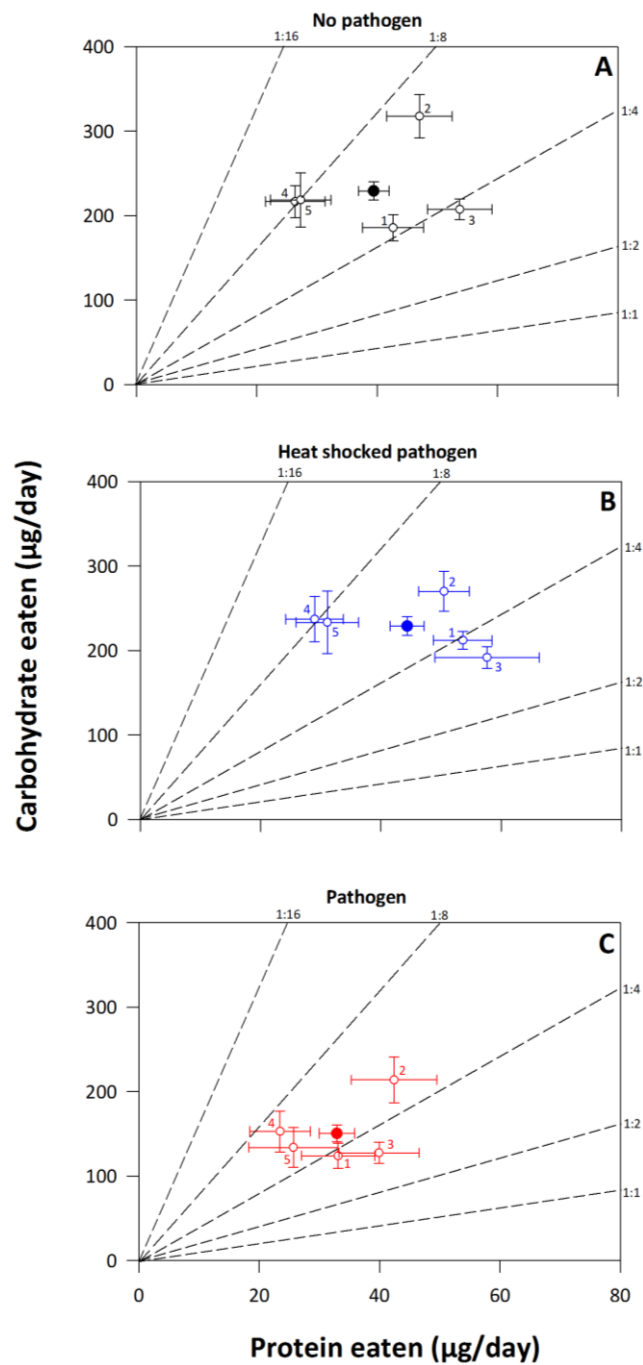


Figure S2: Regulated daily intake points for flies on diet choice for each of the diet pairs (1-5; \pm SE) for **A** untreated flies, **B** pathogen challenged (heat shocked pathogen) flies, and **C** infected flies.

Hormesis results in trade-offs with immunity

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HORMESIS RESULTS IN TRADE-OFFS WITH IMMUNITY

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Many have argued that we may be able to extend life and improve human health through hormesis, the beneficial effects of low-level toxins and other stressors. But, studies of hormesis in model systems have not yet established whether stress-induced benefits are cost free, artifacts of inbreeding, or come with deleterious side effects. Here, we provide evidence that hormesis results in trade-offs with immunity. We find that a single topical dose of dead spores of the entomopathogenic fungus, *Metarhizium robertsii*, increases the longevity of the fruit fly, *Drosophila melanogaster*, without significant decreases in fecundity. We find that hormetic benefits of pathogen challenge are greater in lines that lack key components of antifungal immunity (*Dif* and *Turandot M*). And, in outbred fly lines, we find that topical pathogen challenge enhances both survival and fecundity, but reduces ability to fight off live infections. The results provide evidence that hormesis is manifested by stress-induced trade-offs with immunity, not cost-free benefits or artifacts of inbreeding. Our findings illuminate mechanisms underlying pathogen-induced life-history trade-offs, and indicate that reduced immune function may be an ironic side effect of the "elixirs of life."

KEY WORDS: *Drosophila melanogaster*, ecological immunity, fitness, hormesis, life-history evolution, trade-offs.

Can organisms have it all? One of the central principles of life-history theory is that, because they are constrained by resource limitations, organisms cannot simultaneously optimize all aspects of fitness (Kirkwood 1977; Stearns 1992; Zera and Harshman 2001; Roff 2002; Zuk and Stoehr 2002). This premise has been challenged by studies reporting positive genetic correlations between fitness traits (Spitze 1991; Reznick et al. 2000; Hutchings 2006; Brzek and Konarzewski 2007; Koenig et al. 2009; Schroderus et al. 2012), studies revealing that longevity and fecundity can be decoupled with molecular genetics (Flatt 2011; Kenyon 2011), and studies documenting hormesis, which occurs when low doses of stress-inducing physiological treatments, such as heat shock, diet composition, and toxic chemicals, enhance traits associated with fitness (Minois 2000; Merker et al. 2001; Cypser and Johnson 2002; Hercus et al. 2003; Calabrese 2005; Gems and Partridge 2008; Hunt et al. 2011).

Research on hormesis demands attention from an evolutionary perspective (Forbes 2000; Costantini et al. 2010). Although it is not yet clear whether hormesis acts on Darwinian fitness, thousands of studies have documented the beneficial influence of stressors on important fitness traits, including longevity and fecundity

(Calabrese 2005; Gems and Partridge 2008). Studies identifying beneficial influences of stress on fitness would challenge our understanding of evolution because it would imply that life histories are generally suboptimal (Forbes 2000). Still, with a handful of exceptions (Maynard Smith 1958; Krebs and Loeschcke 1994; Lane et al. 1996; Markowska 1999; Le Bourg et al. 2000; Sørensen et al. 2007), few studies have tested whether physiological treatments that extend life come at a cost to other aspects of life history, particularly in the ability to fight off live infections.

There are two lines of evidence implicating a link between hormesis and immunity. First, although parasites usually reduce the reproductive output and survival of their hosts (Lehmann 1993), a growing body of work shows that animals challenged with dead or even live pathogens exhibit improvements in specific aspects of their life history (Polak and Starmer 1998; Chadwick and Little 2005; Ikeda et al. 2007). In some animals, pathogen challenge increases resistance to subsequent infections (a pattern referred to as immune priming, see Pham et al. 2007; Lawnczak et al. 2007; Roth et al. 2009); but, in other animals, it enhances aspects of physiology, often to the detriment of their ability to fight off subsequent live infections (Leroy et al. 2012; Papp et al.

2012; Ermolaeva et al. 2013). Although it is unknown whether the physiological benefits of pathogen challenges fulfill the characteristic pattern of hormesis (an inverted “U” dose–response relationship with beneficial effects at low doses and toxic effects at high doses), the finding that life-history traits can be improved by a single dose of pathogen challenge suggests that hormesis can be induced by host responses to pathogen challenge (Leroy et al. 2012; Papp et al. 2012; Ermolaeva et al. 2013).

Second, even when the source of stress response is not a pathogen, hormesis appears to be driven by the expression of genes associated with immunity (Calabrese et al. 2012). Heat shock proteins not only contribute to heat shock induced increases in life span (Tatar et al. 1997; Kristensen et al. 2003), but also interact with components of the innate immune system (Chen and Cao 2010). Additionally, the NF- κ B innate immune gene *Dif* has been shown to influence the hormetic benefits of cold shock (Le Bourg et al. 2012) and its expression is known to have a regulatory role in life-history trade-offs between longevity and immunity (Gosselin and Abbadie 2003; Rea et al. 2005; Mattson and Meffert 2006; Lemaître and Hoffman 2007; Salminen et al. 2008; Pursall and Rolff 2011; Chirumbolo 2012; Gartner and Akay 2013). Other work indicates that the activation of innate immunity in response to pathogen challenge is linked both to enhanced physiology and reduced ability to fight off subsequent infections (Papp et al. 2012; Ermolaeva et al. 2013).

Still, the evolutionary implications of hormesis are unresolved (Forbes 2000; Costantini et al. 2010). Does hormesis occur in outbred lines or is it only an artifact seen in nearly isogenic lines, as appears to be evident in diet-restricted, inbred mice (Liao et al. 2010)? Does hormesis represent a switch of life history, promoting survival in the detriment to other traits as suggested for calorie restriction (Tatar et al. 2003)? Does the expression of immune and stress genes generally facilitate or suppress enhancements in longevity? Does it make sense to recommend low-level stress as a therapy for human health, as some have done (Gems and Partridge 2008; Rattan and Demirovic 2009; Vaiserman 2010; Calabrese et al. 2012)? Or, does hormesis inevitably lead to trade-offs with immunity? These are important questions to resolve not only because they relate to how animals fight off infections and whether we can use stress treatments to improve health, but also because they provide a crucial test of the evolutionary principle that life-history optimization is constrained.

Here, we address these questions using the fruit fly, *Drosophila melanogaster* as host for the insect-generalist entomopathogenic fungus, *Metarhizium robertsii* (for further details, see Gao et al. 2011; Zhong et al. 2013). This system has several features that make it suitable for studies of pathogen-induced fitness trade-offs and the age-specific genetic effects, which underlie hormesis: topical application of dead *Metarhizium* spores

is known to stimulate immune responses in insects (Xia et al. 2001). Flies can be challenged by the fungal pathogen en masse by briefly agitating them in flasks with live or heat-killed fungal spores (Zhong et al. 2013). The fruit fly is well suited for large-scale experimental demography (Kohler 1994; Priest et al. 2002). By employing the *Drosophila* RNAi knockdown and mutant knockout lines in conjunction with appropriate control lines, we can assess the consequence of immune- and stress-response gene expression on longevity. Furthermore, we can also investigate the effects of pathogen treatment on life-history patterns in outbred laboratory lines to eliminate the possibility that hormesis is a side effect of inbreeding.

Our central hypothesis was that hormesis trades-off with immunity. This leads to the predictions that hormetic responses to stress should be greater in animals lacking functional immune responses and that hormesis should increase susceptibility to infection. To test these predictions, we used the following methodology: (1) we used an isogenic mutant stock of flies, *w¹¹¹⁸*, to investigate the dose–response relationship between topical exposure to heat-killed fungus and resistance to heat stress. This established that a single dose of topical pathogen challenge was sufficient to induce hormetic benefits. (2) We studied a knockout mutant of *Hsp83* and used RNAi to down regulate three genes, *Dif*, *Turandot M*, and *Turandot C* (all derived from the *w¹¹¹⁸* background), to test how the expression of immune and stress genes contributes to the fitness benefits of pathogen challenge. (3) Employing two outbred laboratory lines, we tested for pathogen-induced trade-offs between survival, fecundity, and susceptibility to subsequent live infections.

We chose the mutant and outbred laboratory lines for specific reasons. We investigated *Dif* because it is a key component of the Toll pathway, which confers antifungal immunity and is a putative regulator of hormesis (Le Bourg et al. 2012); *Turandot M* because it is upregulated in response to infection and provides protection against sexually fungal transmitted infections in flies (Ekengren and Hultmark 2001; Brun et al. 2006; Zhong et al. 2013); *Hsp83* as a positive control because previous work has established that stress-associated molecular chaperones are essential for hormesis (Tatar et al. 1997; Qin et al. 2005); and *Turandot C* because it is upregulated in response to many different types of stress and we had previously established that there was no evidence that it confers immunity to topical fungal infection (Zhong et al. 2013). But, findings in the aforementioned lines could be biased because they are mutant and are derived from an isogenic background. We therefore tested two wild-type lines, *Oregon-R*, a standard outbred laboratory-adapted line maintained in two-week culture (Milkman 1966), and *Dahomey*, another standard laboratory-adapted line maintained in large populations under age-independent culture (Chapman and Partridge 1996).

Methods

FLY AND FUNGUS STOCKS

All experimental animals were maintained at 25°C with 12:12 light:dark cycle in standard *Drosophila* shell vials at low densities (approximately 50 flies/vial) for three generations prior to the start of the experiments. We used an oatmeal-molasses-agar media with added live baker's yeast and an antifungal agent (Nipagin, Sigma-Aldrich, St. Louis, MO), which inhibits the growth of naturally occurring saprophytic fungi.

The *Dahomey* strain of *D. melanogaster* (obtained from Stuart Wigby, University of Oxford) was kept in large population cages (1 m³) with overlapping generations for two years before they were expanded over three generations in low-density culture (approximately 50 larvae/vial). The *Oregon-R* strain (obtained from Tim Karr, Arizona State University) was simultaneously expanded under low-density culture. We acquired the *Hsp83* knockout mutant, Act5C-Gal4 constitutive promoter, and *w1118* background strain on which the knockout and RNAi lines were based from the Bloomington Stock Center. The UAS-*TotM*, UAS-*TotC*, and UAS-*Dif* strains used were originally obtained from the Vienna *Drosophila* RNAi Center, which contained the RNAi constructs for the *Turandot M* and *C*, and *Dif* genes, respectively (for further information, see Dietzl et al. 2007; Zhong et al. 2013). The Gal4/UAS system operates by expressing the RNAi transgene for the target gene through the UAS promoter in all tissues of the fly, driven by the ubiquitous Act5C-Gal4 transcription factor providing universal knockdown of the gene.

We simultaneously generated nine distinct genotypes. We crossed Act5C-Gal4/CyO females with males carrying one of the UAS constructs to generate the genotypes with targeted gene knockdowns and a knockout: Act5C-Gal4/UAS-*TotM*-IR; Act5C-Gal4/UAS-*TotC*-IR; Act5C-Gal4/UAS-*Dif*-IR; and *Hsp83*⁻/*Hsp83*⁻. We crossed Act5C-Gal4/CyO females, UAS construct females, and *w1118* wild-type females to *w1118* wild-type males to generate control genotypes: +/+, Act5C-Gal4/+, UAS-*TotM*-IR/+, UAS-*TotC*-IR/+, UAS-*Dif*-IR/+. Thus, for each gene knockdown, there were three control genotypes (+/+, Act5C-Gal4/+, and UAS-*gene of interest*-IR/+), which permitted being able to account for independent effects of the Act5C-Gal4 promoter and UAS transgenes. The effectiveness of the knockdowns was confirmed by semiquantitative PCR (E. Immonen and M. G. Ritchie, unpubl. ms.). In total, we cultured 2592 vials of flies at 50 ± 10 larvae/vial before the start of the experiment (288 vials/genotype).

Metarhizium robertsii (isolate 2575) was obtained from the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF, U.S. Department of Agriculture). This fungus is a common soil-associated, insect-generalist pathogen commonly used in pest control of large insects (Gao et al. 2011).

We inoculated quarter-strength sabouraud dextrose agar with *M. robertsii* conidia (asexual fungal spores) and incubated the plates at 28°C for four weeks before storing at 4°C for up to three months. Conidia were collected by scraping the surface of the sporulating culture with an inoculating loop. Conidia were autoclaved by placing a large amount of live spores into a glass universal that was taped inside an autoclave bag. This ensured no moisture came into contact with the spores.

METHOD OF PATHOGEN CHALLENGE

Each pathogen challenge treatment involved placing approximately 300 mixed-sex flies of each genotype without CO₂ anesthesia into a 250-ml conical flask with 20 mg of autoclaved (heat-killed) conidia and agitating the flask for 10 sec. Exposed flies were held in temporary holding vials before being transferred to new food vials and into 10 × 15 cm demography cages (see Priest et al. 2002). This treatment method topically inoculates flies at fairly consistent doses of fungus, even after accounting for the effect of grooming on topical dose (Zhong et al. 2013). Sham-treated control flies were manipulated identically by agitating them in an empty flask. The procedure for testing susceptibility to live infection was identical, except that the conidia were not autoclaved before the treatment.

INDUCING HORMESIS THROUGH PATHOGEN CHALLENGE

We first examined how the dose of pathogen challenge influenced resistance to heat stress, to determine whether pathogen challenge fits the inverted "U" (low-level, beneficial) pattern characteristic to hormesis. Flies of the *w1118* (+/+) genotype were collected from eclosion in a 24-h cohort and were left to mature in mixed-sex vials in densities of 50 flies/vial for two days. Following maturation, flies were placed on a 10-day regime where they were exposed to varying frequencies of pathogen challenge using the aforementioned method. The regime consisted of five treatments: 0—no pathogen challenge; 1—a single pathogen challenge on day 10; 1 *early*—a single pathogen challenge on day 2; 3—pathogen challenges on days 2, 6, and 10; and 5—pathogen challenges on days 2, 4, 6, 8, and 10. On days when flies on particular regimes were not exposed to a pathogen challenge, they were conditioned to sham treatment. This ensured that any responses observed were the result of the pathogen treatment. At day 12, all flies were conditioned to heat stress, 38.5°C for 45 min in a water bath, and survival was assayed 20 h posttreatment.

EFFECTS OF PATHOGEN CHALLENGE ON LONGEVITY AND FECUNDITY IN THE W1118 BACKGROUND LINES

We used RNAi and mutant fly lines to assess the influence of stress and immune genes on the pathogen-induced responses on

longevity. For each of the nine genotypes, flies were collected over 24-h cohorts, and held in mixed-sex cages. At day 7, following a randomized experimental design, the flies were either given a sham treatment or single dose of a pathogen challenge. Survival was assessed by recording and removing dead flies every two days post-treatment until all flies perished.

The fecundity of female flies was also assessed in each line to determine whether hormetic effects on longevity led to trade-offs with reproduction. Females were collected as virgins over a 4-h window (immediately subsequent to the original 24-h cohort) and placed in media vials at 20 flies/vial. After two days, 20 *w¹¹¹⁸* *+/+* males were added to each media vial to allow them to mate over a 24-h period. Twenty-four hours after the males had been discarded, females were given a pathogen challenge using the aforementioned method, except females were treated in groups of 20 flies with 6 mg of heat-killed spores. The females were then transferred to single-female food vials and subsequently transferred to fresh food vials every two days for a total of 10 days. Used food vials were held for 18 days after collection at 25°C and then frozen after which the number of hatched pupal cases was counted to estimate fecundity.

TRADE-OFFS BETWEEN HORMESIS AND IMMUNITY IN WILD-TYPE LINES

We used two wild-type outbred fly lines, *Oregon-R* and *Dahomey*, to assess the influence of a single dose of pathogen challenge on survival, fecundity, and susceptibility to live infection. To assess survival, flies were collected over a 24-h period and matured for 48 h in mixed-sex cages. Female flies were given a pathogen challenge on day 4 and dead flies were removed and recorded every two days post-treatment when fresh media vials were supplied. Fecundity was assessed as above, except that the lines were provided males of their own strain and fecundity measures were taken over a total of four days.

To assess the influence of pathogen challenge on immunity, flies were collected over a 24-h period and held for two days in mixed-sex cages to ensure that they were mature prior to the challenge. Following maturation, flies were either given a pathogen challenge or a sham treatment. Two days post-treatment, all groups were treated with live fungal spores. Dead flies were recorded and removed daily following infection until all individuals in the vials were dead. Food was replaced daily.

STATISTICAL ANALYSIS

We used chi-squared contingency tests to investigate the influence of the number of doses of topical treatment with heat-killed fungus on resistance to heat stress. Initially, the proportion alive following heat-stress was assessed across all treatment regimes. Following this, further analyses were completed to compare between individual treatments.

Cox proportional hazard regressions were used to analyze the influence of pathogen treatment on survival and to assess how the treatment responses differed between the genotypes. The full model included genotype, pathogen treatment, and pathogen treatment \times genotype interaction as predictor variables, with age at death as the response variable considered with information on censoring (to account for the small number of flies that escaped during the study). ANOVAs were used to test the significance of interactions between predictor variables by comparing Cox regression models incorporating the interactions with models where they were removed. Separate Cox regressions were performed for each genotype and additionally for each gene of interest that only included the relevant knockdown and control genotypes (e.g., the analysis of *TotM* included the knockdown, *Act5C-Gal4/UAS-TotM-IR*, and the three control lines, *Act5C-Gal4/+*, *+UAS-TotM-IR*, and *+/+*). For each gene of interest, we first estimated the hazard ratios (the change in the probability of death by the next event in pathogen-challenged animals relative to uninfected animals) for the knockdown genotype and also for its combined control genotypes (by pooling raw survival data of the relevant control genotypes) from the Cox models. Sequential Bonferroni corrections were completed on the significance values across the four comparisons. Percentage changes in survival were calculated by inverting the hazard ratio of the genotype to obtain the proportional difference in relation to controls. For heuristic purposes, mean longevity was also estimated for each genotype. A Pearson's correlation was used to assess concomitant changes in fecundity for all genotypes. Additionally, linear models were undertaken to identify the relationship between the effects on survival and fecundity for each knockdown/knockout genotype and their associated controls. The full model included the number of hatched pupae produced as the response variable and genotype, pathogen treatment, and pathogen treatment \times genotype interaction as predictor variables. Sequential Bonferroni corrections were again completed across the four comparisons.

For the outbred wild-type lines, Cox regressions were completed for pathogen challenged relative to sham-treated animals when both infected and uninfected. Linear models were used to assess the fecundity of these populations with total pupae production as the response variable and treatment (pathogen challenged vs. sham treated) as a fixed effect. All analyses conformed to model assumptions and were performed with *R* version 2.15 (*R* Core Team 2012).

Results

A SINGLE DOSE OF TOPICAL PATHOGEN CHALLENGE ENHANCES RESISTANCE TO HEAT STRESS

We found that the influence of pathogen challenge on resistance to heat stress follows the inverted "U" dose-response pattern that

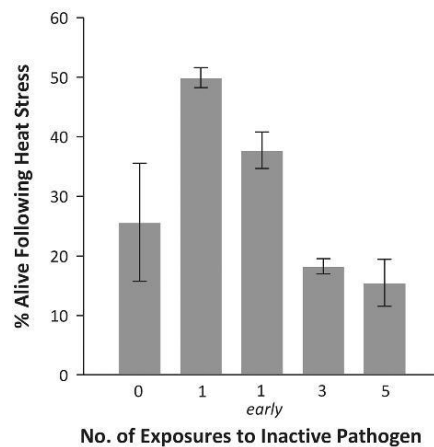


Figure 1. Percentage of flies surviving 20 h post heat stress following topical exposure to heat-killed fungal pathogens at different dose regimes (\pm SE).

is characteristic of hormesis. The number of doses of topical exposure to heat-killed spores influenced variation in resistance to heat stress ($\chi^2_4 = 57.4$, $P < 0.001$; Fig. 1). More specifically, flies that received one dose of pathogen challenge two days before heat stress had increased resistance to heat stress compared with those that received zero ($\chi^2_1 = 7.2$, $P = 0.007$), three ($\chi^2_1 = 24.6$, $P < 0.001$), or five ($\chi^2_1 = 29.0$, $P < 0.001$) doses. We also found evidence that the effect of a single dose on resistance to heat stress is temporary, as animals that received one dose 10 days prior to heat stress (1 *early*) had a nonsignificant difference in their heat stress resistance than that of untreated animals ($\chi^2_1 = 0.3$, $P = 0.576$).

GENOTYPE \times PATHOGEN TREATMENT EFFECTS ON MORTALITY

We found that animals topically challenged with a single dose of heat-killed fungus generally lived longer. The pathogen challenge reduced the relative risk of death by 14% across all lines (Figs. 2A, S1). In the overall model, there was evidence that longevity was influenced by pathogen treatment, genotype, and the genotype \times pathogen treatment interaction (T: $\chi^2_1 = 77.6$, $P < 0.0001$; G: $\chi^2_3 = 3099$, $P < 0.0001$; G \times T: $\chi^2_3 = 64.4$, $P < 0.0001$; Fig. 2B). It was evident that the changes in longevity in response to pathogen challenge were different in the knockdown and knockout genotypes, as removing these genotypes led to nonsignificant G \times T interactions in the full model (T: $\chi^2_1 = 38.1$, $P < 0.0001$; G: $\chi^2_4 = 846$, $P < 0.0001$; G \times T: $\chi^2_4 = 6.3$, $P = 0.178$; Fig. S2).

The longevity benefits of pathogen challenge in isogenic (+/+) *w1118* animals depend on the expression of immunity and

stress genes. While, *Dif* and *TotM* knockdown animals exhibited greater improvements in longevity in response to pathogen challenge than their associated control genotypes (G \times T: $\chi^2_1 = 9.1$, $P = 0.009$; $\chi^2_1 = 30.0$, $P = 0.0004$, respectively; Fig. 2B), *TotC* knockdown flies showed no variation in survival in response to pathogen challenge whereas their control counterparts benefited (G \times T: $\chi^2_1 = 6.4$, $P = 0.024$). There was no evidence of variation in the response to pathogen challenge on longevity in *Hsp83* knock-out animals and their control genotype ($\chi^2_1 = 2.9$, $P = 0.089$).

GENOTYPE \times PATHOGEN TREATMENT EFFECTS ON FECUNDITY

Across all of the lines, we found no evidence that enhanced longevity in response to pathogen challenge came with reductions in fecundity ($t_7 = 2.0$, $P = 0.092$; Fig. S3). There was also little evidence that changes in fecundity in response to pathogen challenges were greater in the knockdowns and knockout than their associated control genotypes (G \times T: *Dif*, $F_{1,323} = 4.3$, $P = 0.156$; *TotM*, $F_{1,333} = 0.2$, $P = 0.701$; *Hsp83*, $F_{1,173} = 0.1$, $P = 0.759$; *TotC*, $F_{1,334} = 2.4$, $P = 0.126$).

TRADE-OFFS BETWEEN HORMESIS AND IMMUNITY IN OUTBRED LINES

Pathogen challenge can generate trade-offs between survival, reproduction, and immunity in outbred lines of flies. In the *Dahomey* line, we find that, in comparison to untreated animals, pathogen-challenged animals had higher survival, higher reproductive output, but also higher susceptibility to live infections ($\chi^2_1 = 12.4$, $P < 0.001$, $F_{1,134} = 12.7$, $P < 0.001$, $\chi^2_1 = 9.0$, $P = 0.003$, respectively; Fig. 3). Note that the 10% increase in fecundity resulting from pathogen challenge in the *Dahomey* line was confirmed in an independent study ($F_{1,161} = 8.4$, $P = 0.005$). The responses in the *Oregon-R* line to a pathogen challenge were similar for survival, fecundity, and susceptibility to live infection, although the survival was not significantly different ($\chi^2_1 = 1.9$, $P = 0.171$; $F_{1,427} = 5.1$, $P = 0.024$; $\chi^2_1 = 6.3$, $P = 0.012$, respectively; see Table S1 for mean values).

Discussion

Based on substantial documentation of hormesis (Calabrese 2005), many contemporary scientists have argued that we may be able to employ treatments that incur low-level stress as therapies for extending longevity and enhancing health (Gems and Partridge 2008; Rattan 2008; Kahn and Olsen 2009; Rattan and Demirovic 2009; Vaiserman 2010; Calabrese et al. 2012). This is an important issue to address not only for its implications for public health, but also for our understanding of life-history evolution (Forbes 2000; Costantini et al. 2010). Our study shows that there can be immunological costs for treatments that extend life.

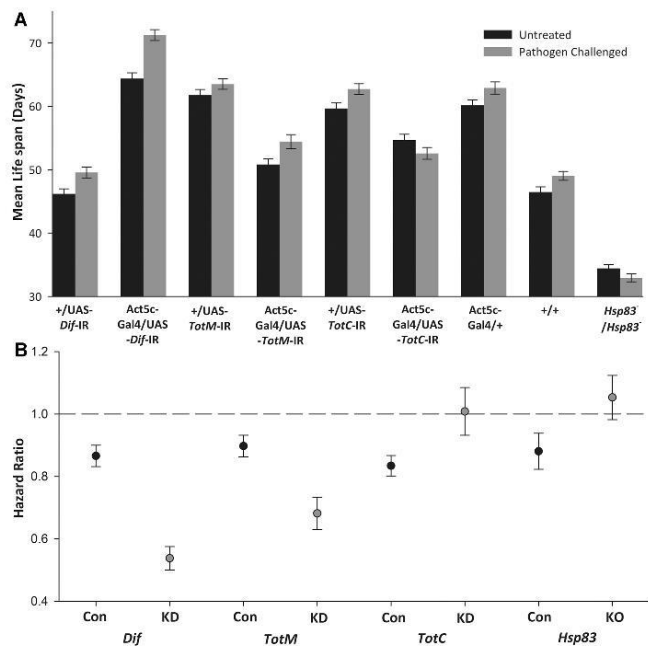


Figure 2. (A) Mean longevity of each genotype. Black indicates flies treated with a sham treatment (control) and gray indicates pathogen-challenged flies (\pm SE). Average life spans were not used in the original analysis as mortality patterns did not meet the assumptions of ANOVA investigation. (B) The influence of the topical exposure to a heat-killed fungal pathogen on the relative hazard ratio parameters to untreated flies (dashed line). Cox hazard proportions are shown for knockdown (KD) and knockout (KO) mutants in gray, and their associated pooled control genotypes (Con) in black (\pm SE). Values under the dashed line indicate that pathogen-challenged animals have increased survival; values above indicate that pathogen-challenged animals experience reduced survival.

Although the induction of hormesis by topical challenge with a dead pathogen may seem unusual, our findings are similar to other described cases of pathogen-induced improvements in physiology (Polak and Starmer 1998; Chadwick and Little 2005; Leroy et al. 2012; Papp et al. 2012; Ermolaeva et al. 2013). The 18% average decrease in the hazard ratio of pathogen-challenged animals observed across all wild-type and control genotype lines is also comparable to the beneficial influences of other stress treatments on longevity in fruit flies, including 5% and 10% from heat stress (Khazaeli et al. 1997; Hercus et al. 2003), 15% from hypergravity (Le Bourg and Minois 1997; Le Bourg et al. 2000); 30% from spermidine (Eisenberg et al. 2009), and 13% and 9% from cold stress (Le Bourg 2007; Le Bourg 2012).

Most of the previous work on the life-extending properties of hormesis has focused on phenomenology, that is, how, but not why, organisms benefit from stress (Forbes 2000; Gems and Partridge 2008; Rattan 2008; Calabrese et al. 2012, although see Costantini et al. 2010). Our results are consistent with the idea

that animals shift their life histories in response to environmental stress (Tatar et al. 2003). We found that topical exposure of dead fungal spores changes a number of key life-history traits and that immune and stress gene expression in the host alters the longevity benefits of the pathogen challenge. We also documented hormetic responses in both genetically mutant isogenic lines and outbred laboratory lines, which indicates that hormesis is not simply an artifact of inbreeding (Liao et al. 2010; Nakagawa et al. 2012). Our findings provide clear evidence that stress genes facilitate and immune genes suppress hormesis, which is in line with previous studies of the genetic basis of hormesis (Kristensen et al. 2003; Mattson 2010; Calabrese et al. 2012; Le Bourg et al. 2012; Gartner and Akay 2013).

It is not clear whether mild exposure to stressors enhances Darwinian fitness or alters one aspect of fitness at a cost to another (Forbes 2000; Costantini et al. 2010). There are a handful of studies that indicate that hormetic benefits on longevity are temporary (Wu et al. 2008) and come with trade-offs in fecundity

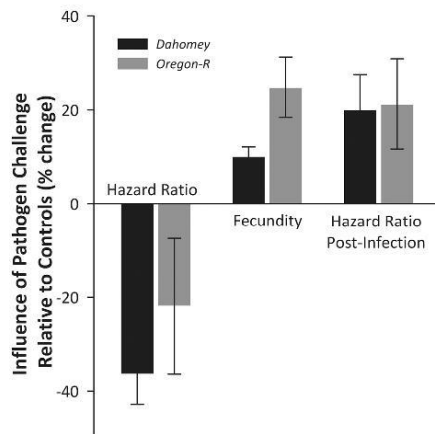


Figure 3. Evidence that pathogen-challenged flies experience increased survival, increased fecundity but increased susceptibility to infection in outbred lines. Estimated %change in trait values are reported \pm SE. Dahomey line is indicated in black; Oregon-R line is indicated in gray.

(Maynard Smith 1958; Krebs and Loeschcke 1994; Lane et al. 1996; Markowska 1999; Le Bourg et al. 2000; Sørensen et al. 2007). But, many studies show that mild exposure to stressors can simultaneously improve survival and fecundity (see Costantini et al. 2010) and it is often presumed that hormetic benefits are cost free (Rattan and Demirovic 2009; Calabrese et al. 2012). Our findings are consistent with the trade-off explanation for hormesis. We found that hormetic responses to stress were greater in animals lacking expression of immune genes *Dif* and *TotM*, which we had previously established provide protection against direct or sexually transmitted fungal infections, respectively (Zhong et al. 2013). Additionally, we found that although pathogen challenge simultaneously increases survival and fecundity, it leads to trade-offs with immunity. These results mirror the finding that the activation of transcription factor SKN-1 in nematodes enhances resistance to oxidative stress and longevity, but increases susceptibility to infection (Papp et al. 2012).

Studies of immune priming have reported that both dead and live pathogen challenges increase, not decrease, susceptibility to subsequent infections (Lawniczak et al. 2007; Pham et al. 2007; Roth et al. 2009). Although our findings seem to be at odds with this result, it might be plausible that immune priming and hormesis might represent divergent strategies for fighting off infections. When they are infected with a lethal pathogen, it is known that animals adopt a myriad of life-history strategies, including fecundity reduction, a long-term strategy that reduces reproduc-

tive output and increases resistance (Hurd 2001), and fecundity compensation, a terminal strategy that temporarily increases reproductive output and decreases immune function (Velando et al. 2006; Weil et al. 2006). It is interesting to note that the dietary and temperature conditions in which we carried the current study are identical to those that lead to a terminal, fecundity compensation strategy in flies infected with a live fungus (V. L. Hunt et al., unpubl. ms.). Thus, it seems plausible that pathogen-induced hormesis might have occurred in our study because flies are mounting a terminal strategy to an infection that never comes.

An alternative explanation for our findings is that hormesis is an artifact of domestication and/or laboratory adaptation (Nakagawa et al. 2012). As we only employed laboratory-selected lines and strains based on isogenic laboratory stocks, our study was not designed to test this question; however, whether hormesis only occurs in domesticated animals does not detract from our findings. The key point is that, when it occurs, hormesis leads to trade-offs with other fitness traits.

Our findings do not necessarily imply that we should ban low-level stress treatments as therapies for human health. It seems quite plausible that in healthy patients, we could employ our natural life-history responses to environmental cues to further improve their health. However, the consequences of hormetic treatments for infected patients could be dire. It is clear that the immunological trade-offs of hormesis need to be identified, acknowledged, and explicitly tested, as others have stated (Gems and Partridge 2008; Rattan and Demirovic 2009; Vaiserman 2010; Calabrese et al. 2012). Further studies of hormesis in humans and model systems could eventually help us identify the selective forces and molecular mechanisms that underlie life-history constraints.

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DATA ARCHIVING

The doi for our data is 10.5061/dryad.85nn2.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Cox hazard proportions of pathogen-challenged flies in relation to their untreated counterparts (dashed line) for each genotype (\pm SE).**Figure S2.** Survival curves for each of the nine genotypes.**Figure S3.** Mean fecundity of each genotype.**Table S1.** Response of trait expression to exposure to heat-killed fungal spores (pathogen challenged) and untreated (control) conditions from the wild-type outbred strains *Dahomey* and *Oregon-R*.

5.8 Supplementary Figures & Tables

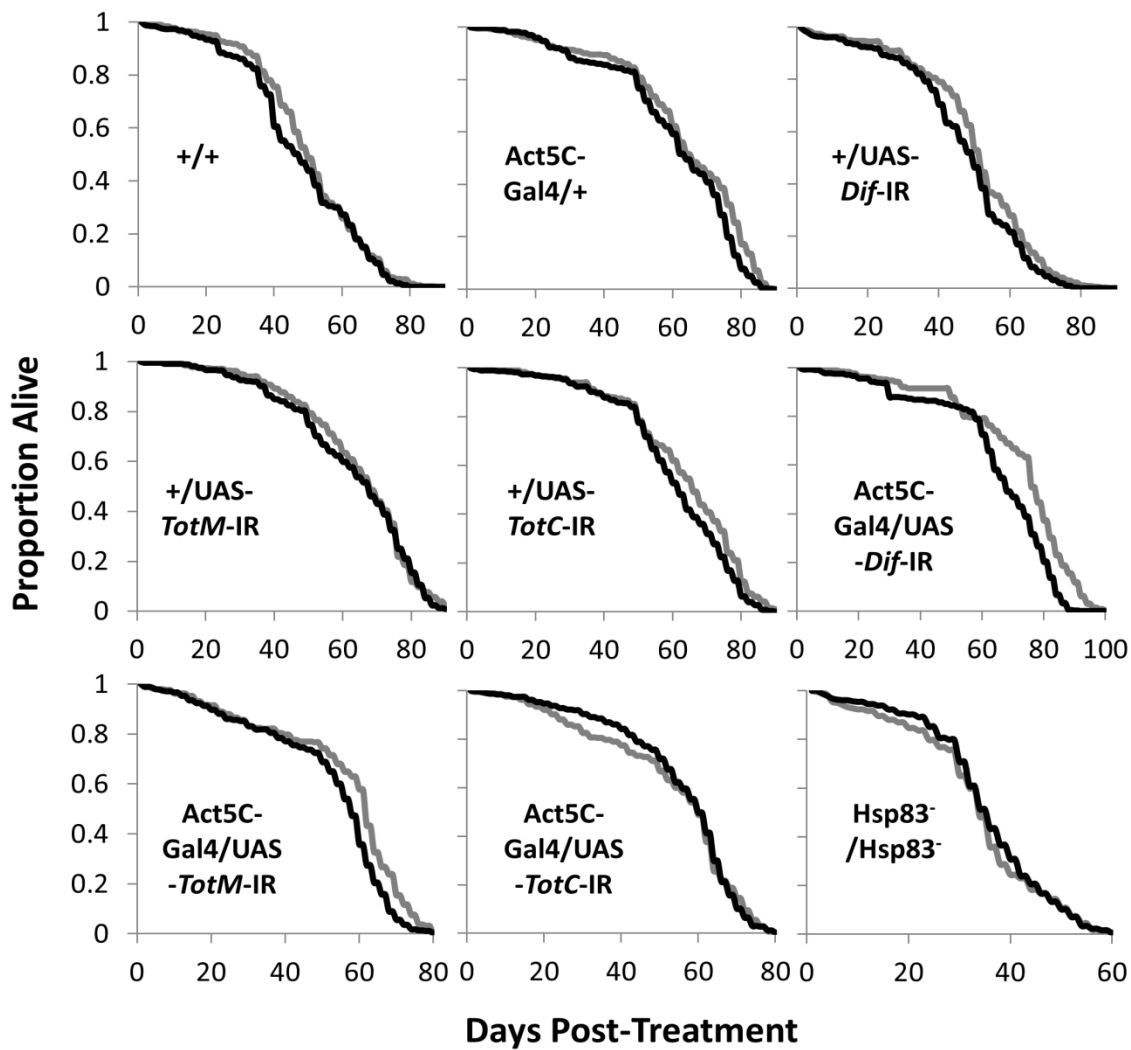


Figure S1: Survival curves for each of the 9 genotypes. Black lines are the survival curves for untreated cohorts while grey lines represent survival curves for pathogen challenged cohorts.

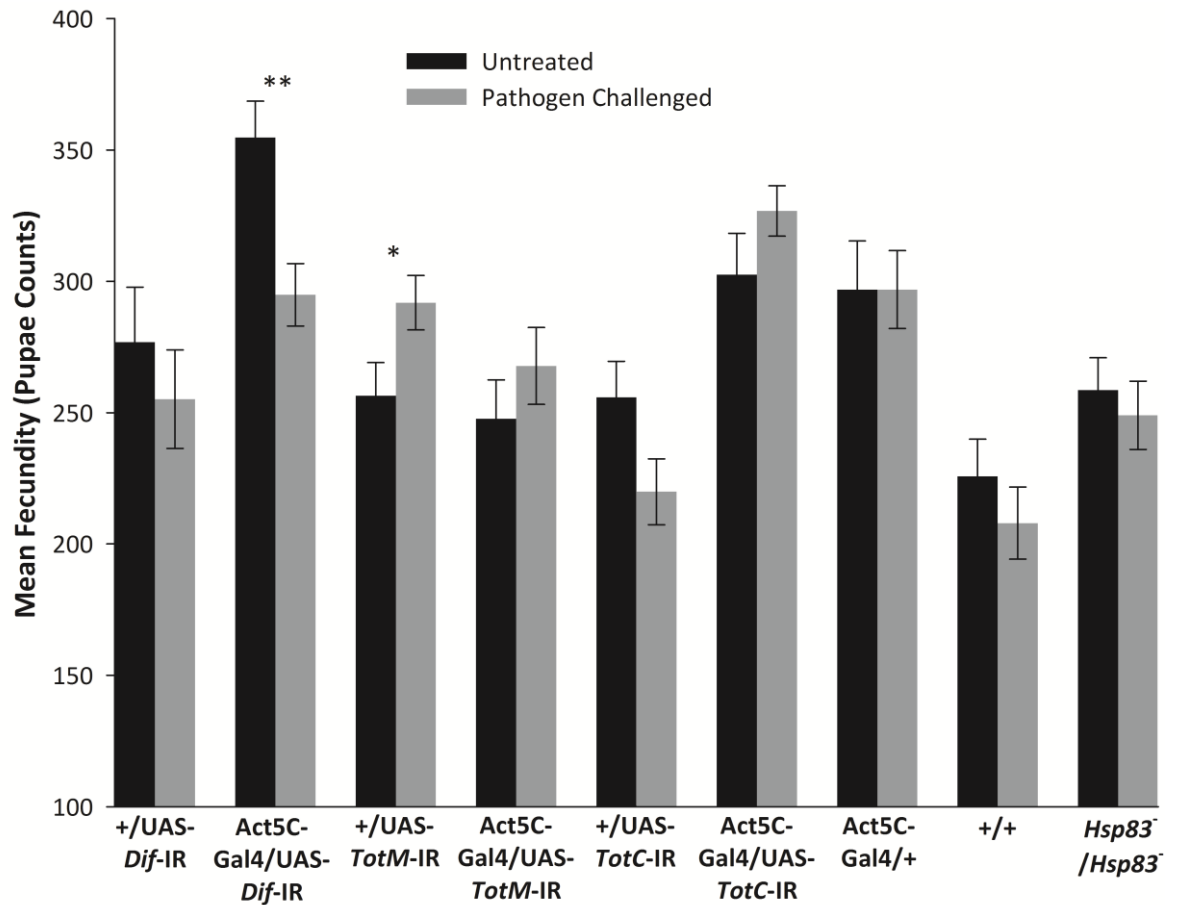


Figure S2: Mean fecundity of each genotype. Black indicates flies treated with a sham treatment (control) and grey indicates flies treated with heat-killed fungal spores (\pm SE). Average fecundity was taken from pupae counts over 10 day samples. * - $P < 0.05$, ** - $P < 0.01$

Table S1: Response of trait expression to exposure to heat-killed fungal spores (pathogen challenged) and untreated (control) conditions from the wild-type outbred strains *Dahomey* and *Oregon-R*. Mean lifespans are given for both uninfected and infected conditions. Mean fecundity under uninfected conditions are shown (\pm SE).

Trait	<i>Dahomey</i>		<i>Oregon-R</i>	
	Control	Pathogen Challenged	Control	Pathogen challenged
Lifespan (Post-treatment)	36.3 (\pm 0.9)	39.7 (\pm 0.9)	38.0 (\pm 2.0)	41.3 (\pm 2.0)
Fecundity	217.6 (\pm 3.9)	239.2 (\pm 4.6)	31.0 (\pm 2.3)	38.8 (\pm 2.5)
Lifespan (Post-infection)	6.9 (\pm 0.3)	6.3 (\pm 0.2)	7.4 (\pm 0.5)	6.5 (\pm 0.4)

General Discussion & Future Work

6.1 Introduction

The overall objective of this thesis is to advance our understanding of how organisms respond to various conditions within nature as well as how & why these vary between individuals. The fundamentals of my approach are that we can gain insights into individual traits and the physiological response by studying the phenotypic plasticity which results from variation in diet and pathogen exposure in the two sexes. The following conclusions can be drawn from my data chapters:

- Male and female *Drosophila* require different nutritional compositions to maximise their reproduction (and thus fitness) but do not differ in their dietary preference
- The nutritional composition of a fruit fly's diet has a pronounced influence on patterns of age-specific mortality and in the ability to suppress pathogen growth
- Infection substantially alters the phenotype-nutrition landscape of the fly which itself, manipulates its dietary composition and intake to alleviate associated fitness costs
- Treatments which enhance lifespan and/or fitness inevitably lead to physiological consequences in other traits

In this chapter, these findings, their implications for physiology, life history and their caveats are discussed as well as potential avenues of further investigation.

6.2 Physiological Implications

In chapter 1 of my thesis, I introduce physiology as the understanding of how an organism functions, and the mechanisms which it employs to survive and reproduce. My thesis has explored the physiological plasticity exhibited in the lifespan and fecundity of males and females to variation in both diet and pathogen exposure. From these analyses, we can identify the reaction norms of these traits, their consequence to the expression of other traits, and how these differ between the sexes. Therefore the results assist in our understanding of the limitations of lifespan, fecundity and pathogen resistance, helping us to identify the way in which resources are used to express these characteristics. Furthermore, acknowledging the physiological differences between the sexes, we can uncover to an extent the sex-specific selective pressures which drive the phenotypic variation between them.

At the beginning of this thesis, I posed important physiological questions which can be addressed through the experiments of my data chapters:

- How does diet affect an organism's ability to survive, reproduce and fight infection?

From the results of chapters 2, 3 and 4, we identify that lifespan is maximised at high concentrations of carbohydrates (i.e. protein:carbohydrate ratio 1:16), reproduction at moderate ratios (particularly within females; i.e. 1:2) and pathogen resistance at high levels of protein (i.e. 2:1). These results highlight the underlying requirements for these various traits such that their dependency on carbohydrates and amino acids differ substantially. Although from previous studies the importance of these macronutrients and available micronutrients in the expression of these individual traits (Bauerfeind and Fischer 2005; Lee et al. 2008; Cotter et al. 2011), the experiments presented here are the first to incorporate holidic diets in an isocaloric or nutritional geometric framework so as to identify the individual effects of calories, carbohydrates and pure protein (i.e. amino acids) on the expression of these traits.

A unique aspect to the studies of chapters 2 and 4 is that lifetime measures of fitness are calculated for each individual. This level of investigation enables us to assess not only how the individual traits of lifespan, fecundity and pathogen resistance respond to variation in diet availability and composition, but also the optimum phenotype which should be selected to maximise fitness in the given environment. Therefore, for the first

time, we can truly identify the specific nutritional requirement of fruit flies to maximise their fitness in both a healthy and infected condition.

- How do these responses and an organism's dietary preference vary with sex?

In chapters 2 and 3, the effect of dietary composition is assessed on various traits between the sexes. Although, in concordance with other studies, we identified that lifespan increased with higher carbohydrate concentrations in both sexes at different rates (Maklakov et al. 2008), we found maximum lifespan not to differ between the sexes. Intriguingly a difference was identified in chapter 3 at a protein:carbohydrate (P:C) ratio of 1:8, which may not have provided sufficient amino acid restriction to obtain the maximal lifespans as expressed in chapter 2 at 1:16.

Interestingly the finding that the dietary composition to maximise lifespan is the same in male and female *Drosophila*, suggests that the differences we observe between the dietary requirements between the sexes are driven by their reproductive effort. We identify that female fecundity is maximised at a higher P:C ratio than males (1:2 vs. 1:8 respectively). This is expected as females are often required to invest greater amounts into the production of their gametic cells than do their male counterparts (Bonduriansky et al. 2008). Therefore under healthy conditions, we expect females to make a greater compromise in dietary preference between maximising their lifespan and fecundity (and therefore fitness) than males. Intriguingly we find no difference in the nutritional preference between the sexes in chapter 2 implying that neither sex is able to acquire the dietary components required to maximise their fitness. This result suggests that the genes underlying nutrient regulation, such as those in the nutrient sensing insulin/insulin-like growth factor signalling (IIS) and target of rapamycin (TOR) pathways (Partridge et al. 2011), may be under intralocus sexual conflict, however additional experiments must be undertaken to assess whether the sexes share a common genetic basis for their dietary preferences (Bonduriansky and Chenoweth 2009; see chapter 2).

Chapter 3 investigates the effect of P:C ratio on pathogen resistance in each of the sexes. Interestingly, although no sex-differences were observed at high protein concentrations (i.e. P:C ratios 2:1 & 1:1), females were found to have lower rates of survival when infected than males at lower P:C ratios. In conjunction with this result, we also identified that females had higher CFU loads than males at these diets. Although this

data is inconsistent with a proportion of the sex-specific immunity literature (Nunn et al. 2009), the patterns of immune performance and pathogen resistance between the sexes are at best equivocal and thus require greater attention. Overall it is clear that females experience a stronger requirement for protein than males due to their necessary investment in their costly-gametic cells, influencing their fitness-optimal phenotype.

- Does pathogen infection alter the nutritional demands and preferences of the host?

Chapter 4 remains the only study to have assessed the dietary effects of infection on the survival and reproduction of the host using nutritional geometry. From these experiments, infected individuals are found to experience drastically different nutritional-trait optima, such that both lifespan and reproduction are maximal at lower calorie and higher protein concentrations than experienced in healthy flies. Therefore infection increases the demand of protein within the animal, while providing selection against ingesting high levels of calories, thus altering the nutritional requirement of the host substantially. Likewise, the preference of infected flies differs from those infected such that they ingest fewer calories and a higher P:C ratio (1:5.81-1:4.57 respectively). Although previous studies have strongly suggested that animals increase their intake of proteins and reduce their overall intake from infection, no investigations have assessed the effects of this dietary alteration on the host's fitness until now (Cotter et al. 2011). Here we find that these behaviours are adaptive to the host, alleviating to an extent the fitness costs of pathogen infection.

- Are the physiological benefits of dietary-restriction induced by calorie or nutrient changes and are they sex-specific?

Much research has been undertaken to determine the causes and effects of dietary restriction on longevity. Although initial dietary manipulation studies concluded the effect was due to calorie restriction (Partridge and Brand 2005), more recent studies challenge this view suggesting it is the limitation of protein, particularly the amino acid methionine, which accounts for this physiological response (Grandison et al. 2009; Lee et al. 2014). The results of chapters 2 and 4 support the latter view as maximum lifespan was obtained at a P:C ratio of 1:16 at relatively high calorie concentrations.

Interestingly, although previous studies suggest a significant difference between the effects of dietary restriction in the lifespans of males and females, we did not find evidence for this (Bonduriansky et al. 2008). Despite identifying a suppressed increase in male lifespan relative to females with decreasing P:C ratio, the maximal lifespan reached did not differ between the sexes. An intriguing finding, and one supported by previous studies, was that under 'moderate' dietary conditions (P:C ratios 1:2 – 1:8), males experienced a higher rate of ageing than females (Chapter 3; Regan and Partridge 2013). It would be insightful to assess whether the ageing rates of the sexes conform at lower P:C ratios (i.e. 1:16), however such investigations have yet to be undertaken.

- Do life-extending treatments incur 'hidden' physiological consequences?

The purpose of chapter 5 was to assess the physiological effects and potential mechanisms of hormesis, a life-enhancing response induced by many low-level stress treatments (Gems and Partridge 2008). Although few, if any, physiological consequences of hormesis have been identified (Le Bourg 2012), here we find that pathogen-induced longevity and fecundity benefits come with costs to fungal-resistance in the fruit fly. This result suggests that all physiological responses have consequences for other traits which should be taken into consideration for future studies of reaction norms. Furthermore, this implies that conditions highlighted as potential treatments to improve particular aspects of life in humans may come with detrimental effects of which we need to be aware.

6.3 Caveats and Considerations

Although our results have important implications for the understanding of the physiological response and phenotypic plasticity of lifespan, fecundity and pathogen resistance between and within the sexes, there are a number of potential limitations and considerations which need to be realised. Firstly, it should be highlighted that all effects have been identified in a single species, *D. melanogaster*. As traits can be significantly genetically determined, the physiological responses which we have found in fruit flies and the variation observed between the sexes, may be specific to this species. Acknowledging this however, particular responses have been identified in a range of species and classes. The longevity benefits observed through dietary restriction and hormesis have been found in a variety of organisms, including mammals (Gems and Partridge 2008; Nakagawa et al. 2012), and the variation between the sexes are well established and supported by our results (Regan and Partridge 2013).

Another concern along the same vein is the use of single pathogen, *M. robertsii*. As discussed in chapter 1, various forms of pathogens (i.e. Gram negative and gram positive/fungal/viral particles) induce particular immune responses in the *Drosophila* host (Lemaitre and Hoffman 2007). Furthermore, the virulence of the pathogen is an important consideration to the physiological consequences of the host and varies at the strain, if not the population, level. Thus the use of a single pathogen can limit our results such that the results observed could be species-specific. Despite this concern however, the majority of the results of this thesis, particularly increased pathogen resistance with increased protein concentration, infection-induced dietary adjustments, and pathogen-induced hormesis, have been identified in other host-pathogen models (please see chapters 3, 4 and 5 respectively). The fact that the pathogen-host responses observed have been identified in other species models ensures that our results have across-species implications, however, the extent and plasticity of these responses could be specific to the *Drosophila-Metarhizium* system employed.

A final caveat of our data relates to the specific environmental conditions applied to our populations within the laboratory. The physiological responses observed were found under a favourable temperature of healthy fruit flies (i.e. 25°C; see SI) and a single humidity (60%; excluding chapters 2 and 4 which were undertaken at 80%) and light:dark cycle (i.e. 12:12). Although these conditions seem moderate and appropriate for

Drosophila culture, these abiotic environmental factors are expected to fluctuate significantly within natural conditions. As the majority of experimentations are undertaken under the favourable abiotic conditions of the study species, it is difficult to assess whether these physiological patterns are experienced in natural environments. Adding to this concern is preliminary evidence from pilot studies which suggest that the pathogen-induced physiological benefits observed in chapters 3 and 5 are not experienced in fruit flies held at unfavourable conditions (i.e. 22°C).

The disparity between laboratory and natural-conditions could be a substantial issue for the behavioural observations of dietary preference assessed in chapters 2 and 4. Aside from the temperature, humidity and day cycles imposed upon these animals, the holidic diets prepared were in liquid form which may have substantial deleterious effects on lifespan and reproduction (please see chapters 2, 3 and Lee et al. 2008 for comparison). Although the results of chapters 2 and 4 are consistent with other studies in sex-specific and pathogen-induced dietary preferences, these investigations have too been undertaken in similar artificial environments. Therefore the implications of these studies, as with those presented here, must be acknowledged with a degree of caution.

6.4 Potential Life History Implications

In addition to implications of these results in understanding the sex-specific physiological responses to diet and pathogen exposure, they also contribute to our knowledge of the life history of the fruit fly and the mechanistics of physiological trade-offs. Life history theory identifies "how natural selection should shape the way organisms parcel their resources into making babies" (Reznick 2009). The way in which organisms "parcel their resources" indicates how individuals share their finite resources (i.e. trade-off) between each of their traits. The overall investment pattern in which an organism employs is termed a life history strategy (Fabian and Flatt 2012) which, although is substantially genetically pre-determined (Holtby and Healey 1990; Zera and Cisper 2001; Braendle et al. 2011), demonstrates a significant degree of plasticity, allowing organisms to adapt to fluctuations within their environment (Stearns 1989; Sandercock et al. 2005). While the chapters support this and assist in identifying the variation in plasticity between the sexes, the results highlight that organisms can also take advantage of environmental variation to employ specific life history strategies which alleviate the costs of particular stresses (see chapters 4 and SI). This finding has implications for ecology and immunology suggesting that organisms may be adopting different strategies against pathogens in natural conditions than when assessed within set laboratory conditions (Schulenburg et al. 2009). These concerns have been raised in the relatively recent movement of ecological immunity (also termed ecoimmunology) which highlights the need for immunological studies to consider the ecology of the study organism, particularly when assessing the potential fitness costs of infection or immune activation (Rolff and Siva-Jothy 2003; French 2009).

As well as assisting our understanding of the plasticity of life history strategies, this thesis reveals aspects of the mechanisms of resource 'trade-offs' between traits, otherwise termed physiological or phenotypic trade-offs (Zera and Harshman 2001). Traditional physiological trade-off theory suggests that particular traits require investment from a shared pool of resources (Stearns 1992). Furthermore, physiological trade-offs are predominantly considered between traits such as current reproduction and survival (Nur 1988), current reproduction and future reproduction (Candolin 1998), and between number, size and sex of offspring (Smith et al. 1989; Stearns 1989) and are therefore primarily assumed to exist between the investigated traits. Our results support

other findings that particular traits (i.e. lifespan, fecundity and pathogen resistance) demand different resources for their function such that lifespan is purely dependent on carbohydrates while fecundity and pathogen resistance depend upon both carbohydrates and proteins and that these may be sex-specific (Maklakov et al. 2008). Furthermore, from chapter 5, we identify that investments can be shared between traits other than those primarily investigated. Therefore physiological trade-offs appear to be more complex than often considered. These relationships are likely based on a number of common resources and contribute to a number (n) of fitness-influencing traits (thus spanning n -dimensions) each of which exhibiting a specific dependence on the separate individual common resources.

Understanding physiological trade-offs in this manner may provide a new light in which to interpret previous phenomena identified in life history studies. Hormesis, as investigated in chapter 5, is often observed to confer trait benefits with no detectable cost (Gems and Partridge 2008). Furthermore, resource allocations between traits appear to be decoupled in a number of lab-reared and genetically manipulated animal models. Often in these populations, enhanced longevity incurred no costs to fecundity (Kenyon et al. 1993; Flatt 2011; Fabian and Flatt 2012; Chen et al. 2013; Gartner and Akay 2013) and, in selected lines, increased growth rates can correlate positively with reproduction producing 'super' phenotypes (Spitze 1991). A potential explanation for these phenomena is that organisms which can acquire more resources can invest more in multiple traits, however in other environments such 'super' phenotypes suffer (Reznick 2000). The results from this thesis suggest an additional explanation that the investigated traits may rely on a variety of 'common' resources and that trade-offs can be observed in other traits aside from those investigated. Together, these insights can account for the inconsistencies in these observations with fundamental evolutionary trade-off theory.

Although our results have implications for the physiological trade-offs within an organism's phenotype and how this varies between environments and the sexes, it is important to note that the insights gained in understanding the life history evolution of the fruit fly is not so clear. Observations of negative correlations between the expression of traits (i.e. physiological trade-off as seen between lifespan and reproduction in chapter 2, and between survival and immunity in chapters 3 and 5), does not imply a genetic trade-off between these traits such that they are evolutionary 'constrained' by one-

another (Worley et al. 2003; Agrawal et al. 2010). This relationship between physiological and genetic trade-offs has often been over-looked within life history theory and has been an area of much discussion and debate (Stearns 1992; Leroi et al. 1994). To determine whether particular traits exist within a genetic trade-off, any genetic correlations must be established either through the use of sibling analysis or artificial selection (Agrawal et al. 2010). An elegant example of the latter is given by Leroi et al. (1994) who observed the physiological trade-offs between starvation and reproduction in *D. melanogaster* in both control and starvation-selected lines. Interestingly, it was found that the physiological trade-off between these traits did not fully correlate with that identified genetically. Therefore, for the physiological observations in this thesis to translate into evolutionary trade-offs of lifespan, fecundity and pathogen resistance, further investigation must be undertaken to determine the genetic correlations and relationships between these traits.

6.5 Proposed Future Work

Although my thesis provides novel insights into many questions in physiology and life history, they open a range of enquiries to be addressed. Here I present an introduction to a number of the potential avenues of investigation.

6.5.1 Improving the *Drosophila-Metarhizium* host-pathogen system

Although the advantages to using the *Drosophila-Metarhizium* host-pathogen system in physiology and ecological immunity have been highlighted in chapter 1, there are a number of aspects which could be improved to enhance the reliability of the results. First, the infection dose of the pathogen is currently relatively uncontrolled. Although a set weight of fungal spores and fly mass are consistently used for infections (see *Materials & Methods* of chapters 3-5; Taylor and Kimbrell 2007), the extent to which an individual is exposed to fungal spores is expected to vary considerably. This particular concern is highlighted from the results of SII. In this chapter, flies infected through sexual transmission of fungal spores were observed to express an alternative immune pathway to those infected directly. Although it can be hypothesised that these immune pathways are activated in a mechanism of infection (i.e. direct infection vs. sexual transmission), it could be possible that particular immune pathways of *Drosophila* are activated in a dose-dependent manner. Developing a method for infecting flies through cuticle exposure with specific doses would determine whether these responses are mechanism- or dose-specific. A proposed method could be to coat a substrate in which flies are in physical contact in a fungal suspension of known concentration such that the density of live conidia on the substrate could be calculated (Tinsley et al. 2006). This could provide greater control over the dose of infection, reducing variation between individuals while ensuring a 'natural' form of pathogen infection.

6.5.2 The role of nutrient-sensing and stress pathways in determining mortality patterns

A significant problem in the field of gerontology has been the reduction of ageing rates in late age populations, a finding termed late-life mortality plateaus (Carey et al. 1992). Identified in a range of large-scale demographic studies in multiple species, including humans, the reduction of mortality risk in late-age has thus far been unexplained (Chen et al. 2013), although a number of theories have arisen to account for these phenomena.

The first proposes that a base level of heterogeneity exists between individuals which results in individual-specific ageing rates (Vaupel et al. 1979). This ‘hidden heterogeneity’ was initially thought to be derived from genetic variation although experiments using isogenic *C. elegans* lines found substantial variation in the rates of ageing (Wu et al. 2006). This suggests that ‘hidden heterogeneity’ has a strong non-genetic component.

A recent study using *Drosophila* attempted to identify the role of diet in explaining this variation. This experiment primarily suggested that the existence and magnitude of the late-age mortality deceleration was due to specific dietary conditions and differed between the sexes (Zajitschek et al. 2014). Furthermore, the link between stress resistance and late-life mortality is also known (Wu et al. 2006). Intriguingly the IIS & TOR pathways are found to underlie the longevity effects of both nutrient limitation and stress resistance (Grandison et al. 2009; Partridge et al. 2011), and are found to vary between the sexes (Regan and Partridge 2013; see chapter 2). It could therefore be proposed that individuals vary in their IIS & TOR expression or sensitivity (potentially through interaction with their gut-residing symbionts; Shin et al. 2011; Ridley et al. 2012) resulting in a variation in life history response to nutrient availability or stressful events producing the heterogeneity in ageing rates which result in late-age mortality plateaus.

A series of experiments could be completed utilising isogenic lines of *D. melanogaster* or *C. elegans* mutant for IIS and/or TOR pathways. These populations could be conditioned to dietary restricted and full feeding environments. The mortality of these populations could be assessed for both males and females separately to identify the existence and magnitude of late-age mortality plateaus. These results could provide conclusive evidence to whether inherent, environmental variation in the expression of the IIS & TOR pathways contribute to ‘hidden heterogeneity’ in ageing rates between individuals and thus late-age mortality deceleration of populations.

6.5.3 The effect of infection-induced dietary adjustment on pathogen growth

A prominent question in the field of immunology and pathology is why do animals reduce their consumption rate when infected (Kyriazakis et al. 1998; see chapter 4). Although chapter 4 provides the best evidence to date that a reduced consumption is an adaptive response increasing fitness when infected, it does not particularly address the effects of host’s behaviour on the invading pathogen. Measuring the pathogen load of flies

conditioned to the 29 diets utilised in the nutritional geometry approach in chapters 2 and 4, would provide sufficient data to assess this question. This study would enable us to ascertain whether adjustments in the dietary preference limits the resources available to the invading pathogen, restricting its growth, or whether it induces physiological changes within the host producing an optimal life history strategy.

6.5.4 Hormesis in natural populations

A concern raised in chapter 5 is that hormesis could be solely expressed in domestic and lab-adapted species and thus may not be a relevant concept to consider in natural conditions and in evolution (Nakagawa et al. 2012). Supportive evidence of this claim lies in the conditional nature of these pathogen-induced physiological responses. As noted previously, additional pilot studies suggest that the longevity and fecundity benefits of the pathogen challenge are not observed in unfavourable conditions of the fly. These results suggest that the benefits of hormetic treatments are only conferred under favourable conditions which would be experienced infrequently within natural environments particularly by poikilotherms. An experimental design to investigate the condition-specific nature of hormetic treatments would be to assess changes in reproduction and lifespan in untreated and pathogen challenged flies under various temperatures in natural lines adapted to a temperature gradient. This study would investigate whether hormesis is induced in natural lines and also whether these physiological responses are specific to the 'favourable' temperature of the fly. A further pilot experiment employing this design was completed in *Drosophila birchii* lines collected across two independent altitudinal lines in Australia and held at 22, 25 and 28°C, with the assistance of Dr. Jon Bridle, University of Bristol. From preliminary data, there was no clear evidence that pathogen-induced physiological responses as identified in chapter 5 were observed in the natural lines. A larger-scale experiment should be completed to provide more conclusive results.

6.6 References

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Adaptive switch in life history strategy driven by cold-seeking behaviour in infected fruit flies

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Abstract

Animals must tailor their life history strategies to suit the prevailing conditions and respond to hazards in the environment. But, how they do this is largely unknown. Here we report that the fruit fly, *Drosophila melanogaster*, optimises its life history by exploiting thermal variation. We find that uninfected control flies prefer warmer temperatures, which facilitates an *r*-like rapid propagation strategy. In contrast, fungus-infected flies prefer colder temperatures, which engenders a *K*-like fecundity reduction strategy that ultimately enhances lifetime reproductive success and resistance to the fungus. These findings help explain how life history trade-offs are mediated and how animals cope with infection in nature, which will be increasingly important given the recent emergence of fungal pathogens and global climate change.

Significance Statement

Animals fight off disease not only by deploying molecular and physiological mechanisms, but also by altering their behaviour. However, we do not understand how these mechanisms are able to simultaneously protect against infection and minimise losses in reproductive output. We show that fruit flies seek out colder temperatures when exposed to a fungal pathogen, which reduces fungal growth and facilitates a shift in reproductive strategy that ultimately increases offspring production. These results are important because they not only demonstrate that life history and temperature preference are flexible, but also that they are integrally linked. These findings suggest that on-going global changes in climate and the spread of fungal pathogens could drastically alter the reproductive ecology of insects in nature.

Running Title: Pathogen-Induced Cold-Seeking Behaviour in Flies

Key words: anapylexia, *Drosophila melanogaster*, *Metarhizium robertsii*, fecundity, longevity, ageing, fitness, mortality rate, fever, behaviour, immunity, ecological immunity.

Introduction

A central premise in evolutionary biology is that life history has evolved to maximise Darwinian fitness (Stearns 1992). Because they can experience radically different environments, even within a single generation, organisms must have the capacity to adjust their schedule of reproduction that best suit the prevailing environment. Despite extensive research on the phenotypic plasticity of life history traits (Stearns and Koella 1986; Gotthard and Nylin 1995; Nylin and Gotthard 1998), how organisms shift their life history strategies in response to environmental change remains poorly understood.

Two of the most important factors shaping the life history of animals in nature are temperature (Huey and Berrigan 2001; Dillon et al. 2007) and parasitism (Minchella 1985; Michalakis and Hochberg 1994; Agnew et al. 2000; Hurd 2001). It is generally thought that animals seek out temperatures which maximise their fitness (Huey and Bennett 1987; Angilletta et al. 2006). Life history theory shows that early-life reproduction has a disproportionate contribution to the intrinsic rate of increase (r), an appropriate measure of Darwinian fitness in age-structured populations, when the population is expanding; but it is substantially less important in stable or declining populations, where late-age reproduction contributes more to fitness (Charlesworth 1994; Brommer 2000). Because the rates of development and early-life reproduction in poikilotherms generally tends to increase with ambient temperature up to their physiological optima (Taylor 1981; Huey et al. 1995; Angilletta et al. 2002; Dillon et al. 2007), we would expect healthy (i.e. expanding) populations to seek out relatively warm temperatures that maximise their intrinsic rate of increase. This prediction has been supported by empirical studies in fruit flies (Martin and Huey 2008) and warm-seeking strains of nematodes (Anderson et al. 2011).

Exposure to pathogens also stimulates shifts in animal life history. Theory generally predicts that parasitised hosts should exhibit fecundity compensation – an increase in current reproductive effort at the expense of reduced lifespan and late-age reproduction (Hochberg et al. 1992; Forbes 1993; Perrin et al. 1996). Another possibility is that infected animals could enhance their Darwinian fitness by fecundity reduction – a decrease in current reproductive effort that ultimately improves survival and lifetime reproductive success (LRS; Hurd 2001). Such shifts in life history in response to parasitism are predicted to occur because there are inherent trade-offs between reproduction and

immune function e.g. the diversion of resources from reproduction to mounting costly immune defences (Sheldon and Verhulst 1996; Schmid-Hempel 2003). However, because pathogens can also actively manipulate the allocation of host resources (Webb and Hurd 1999; Hurd 2003), it is often unclear whether documented shifts in life history represent adaptive changes in the strategy of the host, manipulation by pathogens or unselected by-products of the infection process (Agnew et al. 2000; Hurd 2001).

Studies of pathogen-induced changes in thermoregulatory behaviour could reveal how animals adjust their life history strategy in response to an infection. Infected poikilotherms exhibit both warm-seeking (behavioural fever; Krstevska and Hoffman 1994; Hurd 2003; Kearney et al. 2009) and cold-seeking behaviours (behavioural anapyrexia; Lazzaro et al. 2008; Lazzaro and Little 2009). Because the optimal temperature for r is generally higher than the optimal temperature for LRS across diverse poikilotherms (Huey and Berrigan 2001), we would expect behavioural fever to enhance r and facilitate fecundity compensation, whereas behavioural anapyrexia should enhance LRS and facilitate fecundity reduction. We would also expect the thermoregulatory behaviour to enhance the host's ability to directly suppress pathogen growth and total pathogen burden (i.e. resistance), or improve its ability to mitigate the detrimental impact of infection without directly reducing pathogen growth (i.e. tolerance), or a combination of the two (Schneider and Ayres 2008; Baucom and de Roode 2011; Medzhitov et al. 2012).

Previous studies of pathogen-induced changes in thermoregulatory behaviour have focussed on their impact on the survival and immune function of infected hosts (Müller and Schmid-Hempel 1993; Watson 1993; Adamo 1998; Elliot et al. 2002; Zbikowska and Cichy 2012); however, little attention has been placed on the impact of temperature on reproductive strategy. To address the question of adaptive significance, we need a comprehensive approach, which not only directly measures the consequences of temperature preference on the reproductive output of infected hosts (in terms of both r and LRS), but also of naive control animals and sham-treated animals exposed to inactive pathogens. Here, we employ the fruit fly, *Drosophila melanogaster*, as the host for the entomopathogenic fungus, *Metarhizium robertsii*, to examine the life history and immune consequences of pathogen-induced changes in thermoregulatory behaviour. This system is ideal for this problem: 1) *D. melanogaster* is a model species for the study of life

history (Prasad and Joshi 2003), temperature preference (Dillon et al. 2009), innate immunity (Lemaitre and Hoffmann 2007), resistance and tolerance (Ayres and Schneider 2009), and the interactions between temperature and immunity (Lazzaro et al. 2008; Linder et al. 2008), but the thermoregulatory behaviour of fruit flies during infection is unknown. 2) *M. robertsii* is a common insect pathogen that has previously been shown to induce behavioural fever in locusts (Elliot et al. 2002; Ouedraogo 2003) and drives reproductive and survival costs in fruit flies (SII).

Materials & Methods***Culture maintenance and infection treatment***

With the exception of temperature preference tests, which also used the *Canton-S* and *Tempe-T* strains of *D. melanogaster*, all experiments used the *Oregon-R* strain that had been cultured at 25°C, 40% RH and 12:12 light/dark cycle on standard Nipagin-infused (an antifungal agent) oatmeal-molasses-agar media supplemented with a single grain of live baker's yeast. We cultured *M. robertsii* (isolate ARSEF 2575, previously classified as *M. anisopliae* strain ME1) at 28 °C in continuous light on one quarter strength Sabouraud Dextrose Agar with additional yeast extract (SDAy) and collected spores after 7-14 days. We infected flies by gently shaking cohorts of 10-15 flies in a 250ml conical flask containing 900µg of live spores for 10 seconds. Inoculated flies were sequentially transferred to fresh vials containing standard media to minimise the transfer of excess spores to the experimental vials. Control and heat-killed pathogen treatments were handled identically, except that the flask was empty or filled with 900µg of autoclaved (121 °C, 15 min) spores, respectively.

Temperature preference of Drosophila

We measured temperature preference of flies using on a purpose-built apparatus (Figure S1a). Four escape-proof experimental lanes were created along the length of the apparatus with a perspex lid and the application of the insect deterrent Fluon® to the inner walls encouraged flies to stay on the surface of the aluminium block. A piece of white paper placed on the aluminium block and marked into 10 equal sections was used to identify the position of flies across the gradient. A *k*-type thermocouple (Omega Engineering) was used to measure the mid-point of each section across the apparatus, which confirmed a linear temperature gradient ranging from 16 to 32°C along the aluminium block (Figure S1b).

We transferred control or infected mixed sex flies without anaesthesia in groups of 10-15 flies into the apparatus at 24, 48 and 72 hours post-infection. Temperature preference of each fly was established by recording their locations along the temperature gradient after 30 minutes. All flies were measured only once and the apparatus was cleaned with 70% ethanol after each trial and the paper marking the 10 sections replaced. Temperature preferences were assessed between 11:00 and 14:00 with a minimum of 4

trials for each pathogen treatment/time point. We also assessed the distribution of flies in the absence of thermal gradient at room temperature (Figure S2c).

Effect of temperature on host survival and age-specific mortality

We treated 3-4 day old mixed sex adult flies with live, heat-killed or control pathogen treatment and kept in population cages at 22 or 25°C until all animals had died (11 x 15 cm; n = 50 cages at approximately 40 flies/cage). The cages were provided fresh fly media vials daily and dead flies were removed and recorded. We also confirmed the cause of death by random sampling of cadavers and examining them for signs of *Metarhizium*-like fungal growth. Sampled cadavers were surface sterilised (brief immersion in 1% bleach, 70% ethanol and sterile water) and placed on filter paper moistened with sterile water in sealed Petri dishes. The resulting plates were kept at 28°C for up to 10 days to check for external fungal growth under a dissection microscope.

Life history consequence of temperature preference and infection

Two day old adult virgin females were allowed to mate with males for 24 hours at 25°C in groups of 20 flies. Males were then discarded and females were maintained for a further 24 hours at 25°C. We treated females (adult age 4 day; n = 259) with one of three pathogen treatment (control, live and heat-killed fungus) and transferred them into individual vials containing fresh media in 10 randomised blocks at 22 or 25°C. Every 2 days post-infection, we counted the number of eggs laid by each female and replaced the food media with fresh vials until the fly died. The number of adult deaths was also recorded after each egg collection. We incubated vials containing eggs incubated at 25°C and examined them again after 14 days to record the number of eclosed pupae.

Effects of temperature on fungal growth, host resistance and tolerance

Because host temperature preference could function as a mechanism of resistance (i.e. direct suppression of the growth of pathogens) we assessed the growth rates of *M. robertsii* both on artificial media and live hosts. For *in vitro* growth, we placed 4mm non-sporulating mycelial plugs in the centre of SDAy plates at 22, 25 and 28 °C in continuous darkness (n = 30). We then measured the diameter of fungal mycelium daily along two perpendicular axes drawn on the petri dish for a total of 8 days. We estimated *in vivo*

growth of rate *M. robertsii* from periodic sampling of infected host pathogen load. Mixed sex fruit flies were first inoculated with 20-25 mg of *M. robertsii* according to the infection protocol (11 x 15 cm; approximately 350 flies/cage). Treated flies were then placed at five temperatures ranging from 18 to 28°C over a period of 17 days (n = 30 cages). We randomly sampled pairs of surviving flies on day 3, 5 (28°C treatment only), 7, 10, 14 and 17 post-inoculation for all temperature treatments (n = 147 flies). Sampled flies were individually surface sterilised (brief immersion in 1% bleach, 70% ethanol and sterile water) and homogenised in a buffer of 0.04% Tween®80. The homogenates were spread on fresh SDAY plates and incubated at 28°C for three days after which *M. robertsii*-like colony forming units (CFUs) were identified by spore morphology using a microscope and counted. In cases where the number of CFUs on the plate was too large to count (> 700), pathogen load was estimated by multiplying the mean CFUs within 1cm² sample squares by the number of squares.

We defined host tolerance as the norm of reaction between pathogen load and mortality risk (Simms 2000; Baucom and de Roode 2011). For host mortality rates, we recorded the daily number of deaths from the same fly populations that we sampled flies for estimating pathogen load.

Statistical analysis

All statistical analyses were carried out with SPSS version 13.0 and R version 2.11.1 (R Development Core Team 2010). Planned treatment contrasts were used to assess significance between treatments within full statistical models.

We analysed temperature preference with chi-squared tests by pooling flies from all replicate trials and dividing them into three temperature categories along the temperature gradient (cold, medium and warm). For host survival and mortality, we first fitted Cox proportional hazard regressions with pathogen, temperature and pathogen × temperature using female survival data (models fitted using males gave qualitatively similar results). We then converted the survival data into natural-log transformed mortality rates before fitting age-specific mortality models (Pletcher 1999; Pletcher et al. 2000). The parameters of the age-specific mortality models were estimated using minimised sums of squares and maximum likelihood methods in the R package ‘Survomatic’ (version 1.4.0.0). We combined the sexes for model fitting as we did not did

not find any sex differences in mortality patterns. Individual Gompertz and the more complex logistic models were fitted to each pathogen/temperature treatment combination. Gompertz model describes the classic pattern where mortality increases exponentially with age and has the hazard function ae^{bt} where t is the age at death, a is the morality intercept (background or age-independent mortality), and b is the rate of increase in mortality (rate of ageing). Logistic model modifies Gompertz model by adding the s parameter, the rate of deceleration of mortality at older ages (mortality levelling-off) and is described by the hazard function $ae^{bx}[1 + (a s/b)(e^{bx} - 1)]^{-1}$. Log-likelihood ratio tests were used to assess the significance of the differences between estimated parameter values for all treatments.

We assessed the effect of temperature and infection on two measures of reproductive fitness (LRS and r). LRS was measured as the total number of eclosed pupae over the entire lifetime for each female. r was estimated for each treatment combination within each block using the number of eclosed pupae produced at each collection interval ($n = 30$). We obtained estimates of r by solving numerically the discrete form of the Euler-Lotka equation: $1 = \sum e^{-rx} l_x m_x$, where x is the age class, l_x is the probability of surviving from age class x to age $x+1$, and m_x is the expected number of offspring for a female in age class x (Charlesworth 1994). We then performed separate ANOVAs on LRS and r that included pathogen, temperature, and pathogen \times temperature. To examine the effects of temperature and infection on age-specific fecundity, we fitted separate linear mixed effects models to eggs and pupae data. The full models included eggs or pupae as the response variable; age, pathogen, temperature and all their interaction terms as fixed effects; Individual females were treated as a random effect. We excluded fecundity data in the first two-day interval (day 2-4 post-inoculation) from analysis of age-specific fecundity to improve the model fit. Repeating the analysis with the first interval yielded similar results.

For host resistance, we assessed the effect of host temperature preference on the growth rates of *M. robertsii* both on artificial media and live hosts. For *in vitro* fungal growth, one-way ANOVA was performed on mean daily growth rates on replicate media plates (mm/day). For growth rate within live hosts, we performed 1) linear regression, with a quadratic term, on the estimated growth rates of individual replicate cages up to peak CFU counts ($n = 30$; $\ln(\text{CFU})/\text{day}$); 2) linear regression on the time taken for each

replicate cage population to reach peak CFU counts; and 3) mixed effects model on log-CFU counts over time, with temperature and time as fixed effects and cage as a random effect.

For host tolerance, we used mixed effects models to assess the relationship between pathogen load and host mortality across five temperatures. Significant temperature effect on the slope of the correlation between pathogen load and host mortality (pathogen load \times temperature interaction) would indicate changes in levels of host tolerance i.e. the ability of the host to maintain low mortality despite increasing pathogen load. At each time point, CFU counts obtained from the same cage were averaged and log-transformed. Age-specific mortalities were estimated by calculating the proportion of flies dying on the day of live fly sampling and two days preceding it (i.e. within 72 hours of live fly sampling). The full model included natural log-transformed age-specific mortality as response variable; pathogen load ($\ln(\text{CFU})$), temperature, pathogen load \times temperature as fixed effects; time (number of days post-inoculation) was added as a covariate to account for non-independence of mortality rate estimates over time. Individual cage was fitted as a random effect. An autoregressive error structure was fitted using *corAR1()* function in R package 'nlme' (Pollitt et al. 2012).

Results

Temperature preference of *Drosophila*

When placed on a linear temperature gradient, control flies typically preferred approximately 25°C (Figure S3), but switched their preference to colder temperatures (approximately 22°C) within 24 hours after topical inoculation with live *M. robertsii* spores (Figure 1). This behavioural anapyrexia persisted until at least 72 hours post-inoculation (Live pathogen vs No pathogen control: $\chi^2_1 = 15$, $P = 0.002$ at all time points) and was consistent across multiple laboratory strains of *D. melanogaster* (Figure S4). Interestingly, flies treated with heat-killed spores also exhibited behavioural anapyrexia at 24 and 72 hours post-inoculation, though to a lesser degree than live pathogen treated flies (Heat-killed pathogen vs No pathogen: 24hours, $\chi^2_1 = 8.2$, $P = 0.04$; 72 hours, $\chi^2_1 = 12$, $P = 0.007$).

Effect of temperature on host survival and age-specific mortality

We found that the colder temperature preferred by *Metarhizium*-infected flies extended survival post-infection, but the warmer temperature preferred by control flies elevated their risk of death. As expected, independent survival experiments confirmed that exposure to live fungal spores reduced the survival of flies (Pathogen, $\chi^2_2 = 925$, $p < 0.001$), and that residing at 22°C conferred survival benefits to infected flies relative to staying at 25°C (Temperature, $\chi^2_1 = 214$, $P < 0.001$; Figure S5). However, infected flies do not benefit proportionally more from colder temperature than uninfected control or heat-killed fungus treated flies (Pathogen \times Temperature, $\chi^2_2 = 3.1$, $P = 0.21$). The same pattern was confirmed by age-specific analysis of mortality rates. In contrast to the control and heat-killed fungus treatments, which fitted the simple Gompertz mortality trajectories (Figure S2a & b), the live pathogen treatment significantly increased the age-dependent mortality rate and caused a prolonged period of mortality “levelling off”, justifying more complex logistic mortality models ($\chi^2_1 = 2.7$, $P < 0.05$ for live pathogen treatment at all temperatures; Figure S2c). However, colder temperature was only associated with reduced background (age-independent) mortality, and this effect was found in all pathogen treatments ($\chi^2_1 = 7.8$, $P < 0.001$ for all treatments).

Life history consequence of temperature preference and infection

We found that pathogen-induced behavioural anapyrexia results in an adaptive switch in the life history of female fruit flies from a strategy that favours early-age reproduction and r to one that favours later age reproduction and LRS. Relative to 25°C, flies residing at 22°C generally had lower intrinsic rate of increase ($F_{1,24} = 19.4$, $P < 0.001$; Figure 2a), but higher lifetime reproductive success (Temperature, $F_{1,227} = 4.0$, $P = 0.047$; Figure 2b; Table S1), though the effect on LRS was only significant in the live pathogen treatment (*a priori* contrast, $t = 2.3$, $P = 0.021$). Surprisingly, although exposure to live *Metarhizium* spores greatly reduced total egg production of flies ($F_{2,227} = 132$, $P < 0.001$), we found that overall pathogen treatment did not significantly reduce either LRS or r of infected flies (LRS, $F_{2,227} = 1.1$, $P = 0.34$; r , $F_{2,24} = 2.3$, $P = 0.18$; Table S1).

Age-specific analysis of female reproductive output (eggs) further revealed that temperature and infection had complex effects on the pattern of reproduction in the fruit fly (Age x Temperature x Pathogen: $F_{2,4923} = 16$, $P < 0.001$; Figure 3a-c; Table S2). From a peak at 2-4 days post-inoculation, egg production declined rapidly with age in all treatments (Age, $F_{1,4923} = 218$, $P < 0.001$). Flies residing at 25°C had significantly higher early-age fecundity at the expense of much lower late-age fecundity than those at 22°C (Age x Temperature, $F_{1,4923} = 77$, $P < 0.001$). However, while the fecundity patterns of infected flies were initially similar to the control treatments at the two temperatures (0-2 days post-inoculation), as the infection progressed (after 4-6 days post-inoculation), the decline in fecundity was much slower for infected animals residing at 22°C than those at 25°C (Live pathogen: Age x Temperature, $F_{1,237} = 9.8$, $P = 0.002$; Figure 3c; Table S3).

Fungal growth, host resistance and tolerance

We found that the colder temperature preferred by *Metarhizium*-infected flies enhanced host resistance, but not tolerance to fungal infections. Colder temperatures reduced fungal growth rate both *in vitro* and *in vivo* ($F_{2,15} = 69$, $P < 0.001$; $F_{1,26} = 22$, $P < 0.001$; Figure 4) and increased the time taken to reach peak pathogen load in the live host ($F_{1,18} = 49$, $P < 0.001$). Thus, cold-seeking behaviour is an effective mechanism of resistance against fungal infections. In contrast, we found no evidence that cold-seeking behaviour affected tolerance. The rise and subsequent fall in pathogen load occurred more rapidly at warmer temperatures; consequently, the pathogen load at any particular temperature

depended greatly on the time post-inoculation at which it was measured (Temperature x Age, $F_{4,103} = 3.4$, $P = 0.012$; Table S4). Using mixed effects regression models, we found that while both higher pathogen load ($F_{1,98} = 4.6$, $P = 0.035$) and warmer temperatures ($F_{4,25} = 7.7$, $P = 0.026$) increased the host's risk of death, moving to the colder temperatures did not enhance the capacity of the host to mitigate the harmful effects of fungal infection on mortality (Pathogen load x Temperature, $F_{4,98} = 0.96$, $P = 0.43$; Figure S6; Table S5).

Discussion

There has been extensive interest in understanding how organisms balance the allocation of resources to life history traits (Stearns 1992; Charlesworth 1994). However, the mechanisms by which organisms adjust their life history in response to environmental change, particularly in response to an infection, are poorly understood. It is also unclear whether those changes always result in greater Darwinian fitness. We found that in addition to being an effective mechanism of immunity, pathogen-induced thermoregulatory behaviour functions as a means for rapid and reversible adjustment of host life history strategy. Our findings are important because they show that phenotypically plastic life history responses can be mediated by thermoregulatory behaviour, thereby mitigating the cost of parasitism. We also highlight that measuring classic fitness measures, LRS and r , within the same experiment can yield novel insights (Huey and Berrigan 2001; Anderson et al. 2011).

'Non-immunological' mechanisms such as thermoregulatory behaviour are increasingly appreciated as critical components of an animal's defence against pathogens (Thomas and Blanford 2003; Parker et al. 2011; de Roode and Lefèvre 2012). In contrast to the well-documented phenomenon of behavioural fever (Watson 1993; Adamo 1998; Elliot et al. 2002; Richards-Zawacki 2010), though consistent with other cases of behavioural anapyrexia (Müller and Schmid-Hempel 1993; Zbikowska and Cichy 2012), we found that *D. melanogaster* infected with the fungus *M. robertsii* preferred colder temperatures than uninfected control animals. This switch in temperature preference is likely to be driven by the host, rather than being a result of pathogen manipulation, since even flies treated with heat-killed fungus displayed increased preference for colder temperatures.

Though previous work has argued that behavioural fever and anapyrexia provide survival benefits for infected animals (Müller and Schmid-Hempel 1993; Adamo 1998; Elliot et al. 2002; Richards-Zawacki 2010), we find that survival benefits alone are not sufficient to explain why infected animals prefer colder temperatures. While cold-seeking behaviour indeed enhanced the survival of *Metarhizium*-infected fruit flies, uninfected control flies also received survival benefit by residing at colder temperatures. The survival benefit of colder temperature did not derive from reduced rates of ageing (in control animals) or physiological decline (in infected animals). Instead, lower temperature greatly

reduced the background risk of death, but its influence was roughly equivalent within each pathogen treatment. Thus, if colder temperature provides universal survival benefits, why should uninfected flies prefer significantly warmer temperatures?

Our results suggest that fruit flies exploit their thermal environment to adjust their life history strategies, the pattern of age-specific reproduction. Uninfected poikilothermic animals in expanding populations are expected to favour fast development and early-age reproduction, both of which are positively influenced by ambient temperature (Taylor 1981; Huey et al. 1995; Dillon et al. 2007), in order to maximise their intrinsic rate of increase (Dimbi et al. 2004; Dillon et al. 2009). Previous studies using *D. melanogaster* indicate that adult flies have strong temperature preference at approximately 24-25°C (Sayeed and Benzer 1996; Dillon et al. 2009), we confirmed this in uninfected control flies and found that they achieved higher intrinsic rate of increase at 25°C than those kept at 22°C. This is consistent with previous finding that in *Drosophila* r is maximised at 25°C (Siddiqui and Barlow 1972; Martin and Huey 2008). In contrast, we found that lifetime reproductive success was not significantly different at the two temperatures in uninfected control treatments. Together with the recent study in nematodes (Anderson et al. 2011), our results suggest that at least in some populations of poikilotherms, temperature preference might have evolved to maximise intrinsic rate of increase.

When exposed to pathogens, hosts could adopt fecundity compensation or fecundity reduction strategies (Forbes 1993; Hurd 2001). We found that when they had access to thermal variation, infected animals chose cooler temperatures, which reduced their early age fecundity and r , but enhanced late-age reproduction and LRS, a pattern suggestive of a fecundity reduction strategy. This result is consistent with the observation that the temperature that maximises r is often greater than that which maximises LRS in poikilotherms (Huey and Berrigan 2001). The adaptive values of fecundity compensation or reduction are likely to depend on the demography of the population. In particular, fecundity compensation might be maladaptive declining populations associated with pathogen-rich environments (Charlesworth 1994; Brommer 2000).

Temperature preference alters life history strategies by mediating the trade-off between reproduction and immunity. Although we know immunity has costs which result in trade-offs with other components of fitness (Sheldon and Verhulst 1996; Schmid-Hempel 2003), the mechanisms by which animals mediate these trade-offs to enhance

fitness are poorly understood (Flatt et al. 2005). We found that fruit flies at warm temperatures achieved high early-age reproduction and r at the expense of resistance to fungal pathogen; however, by moving to colder temperatures, they improved their antifungal resistance at a cost to r . Cold-seeking behaviour enhanced resistance against *Metarhizium* infections because colder temperature directly reduced germination success and vegetative growth of fungal spores. Temperature could also have influenced the expression of host immune genes (Linder et al. 2008) and function of haemocytes (Ouedraogo 2003). Interestingly, we found that the colder temperature did not enhance tolerance to fungal infection; defined here as the reaction norm between pathogen load and host mortality risk (Simms 2000; Baucom and de Roode 2011), which suggests that resistance and tolerance can vary independently (Ayres and Schneider 2008). Together, these results suggest that if we only conduct our experiments under a single thermal condition, we may not be able to accurately assess the costs and benefits of immunity (Moret and Schmid-Hempel 2004; Lazzaro and Little 2009).

The principle caveat of these findings is that our design may have augmented the consequences of temperature on life history and immunity. We deliberately controlled for the multitude of factors which influence *Drosophila* temperature preference such as rearing temperature and humidity (Good 1993; Krstevska and Hoffmann 1994; Dillon et al. 2009) by conducting all pathogen treatments simultaneously with uninfected controls under carefully controlled conditions. However, by excluding potential confounding environmental factors we might limit the ecological relevance of what we have found for field situations (Cisarovsky et al. 2012). We also used lab-adapted strains of *Drosophila* strains which could have diverged from wild populations in their life history and temperature preference. In particular artificial culture methodologies could have inadvertently selected for increased early age fecundity (Sgro and Partridge 2000) and higher temperature preference (McDaniel et al. 1995) in uninfected control flies. But, these possibilities are unlikely to influence our conclusions as we identified consistent temperature preferences among lines which have been exposed to different forms of lab adaptation. Lastly, it might appear counter-intuitive that even though live fungal infection greatly reduced total egg production of infected flies, this did not translate into significantly reduced r or LRS based on the production of eclosed pupae. This could be explained by the fact that 1) infection has weak effects on reproduction very early in life

which also contributes the most to measurements of r ; and 2) as mating opportunities were limited to a single 24 hour window at the start of the experiment and female *D. melanogaster* store relatively small numbers of sperm after a single mating, sperm storage was likely to be depleted in uninfected control females resulting in fewer viable offspring (Lefevre and Jonsson 1962).

Taken together, our findings suggest that the exploitation of thermal variation is an important mechanism for poikilotherms to tailor their life history strategies in changing environments. In particular, we hope that these results stimulate further experimental work that directly assess the importance of this mechanism in wild populations where poikilotherms are subject to fluctuating environmental temperatures and pathogenic exposure. It has recently been suggested that behavioural thermoregulation will be a key mechanism for poikilothermic animals to buffer the impacts of global climate change (Kearney et al. 2009; Gvozdík 2012). Moreover, given the recent global spread of fungal pathogens (Fisher et al. 2012), thermoregulatory behaviours are likely to play increasingly important roles in defending against these threats.

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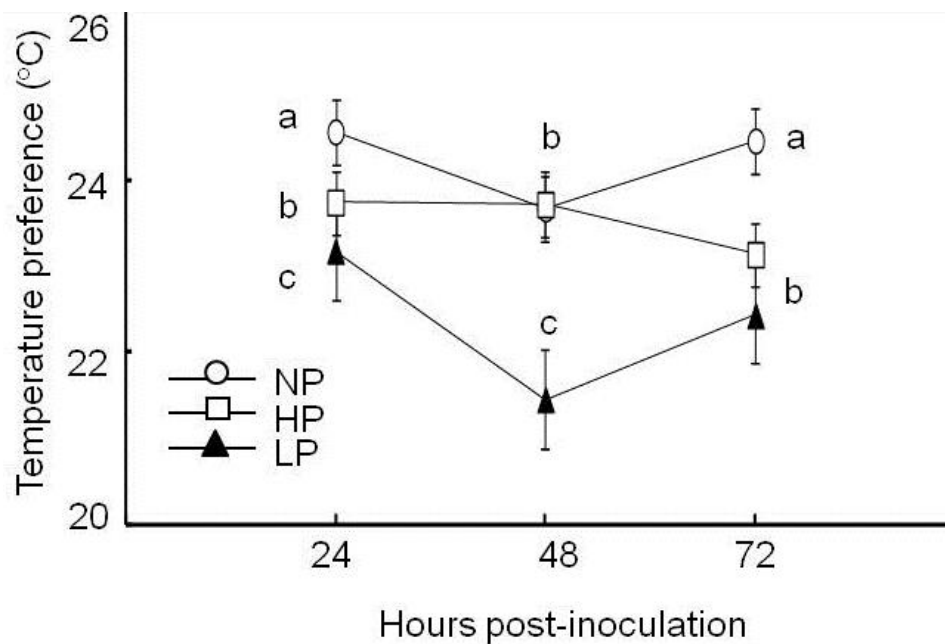
Figures & Tables

Figure 1: *Drosophila melanogaster* infected with *Metarhizium* prefer colder temperature relative to uninfected control animals. Flies were topically inoculated with live *Metarhizium robertsii* spores (LP, triangles), heat-killed *M. robertsii* spores (HP, squares) or no pathogen controls (NP, circles) and placed on a temperature gradient ranging from 16 to 32°C at three time points post inoculation. Within each time point (24, 48 and 72hours), different letters indicate significant difference at $P = 0.05$ (χ^2 test). All data represent mean \pm SE.

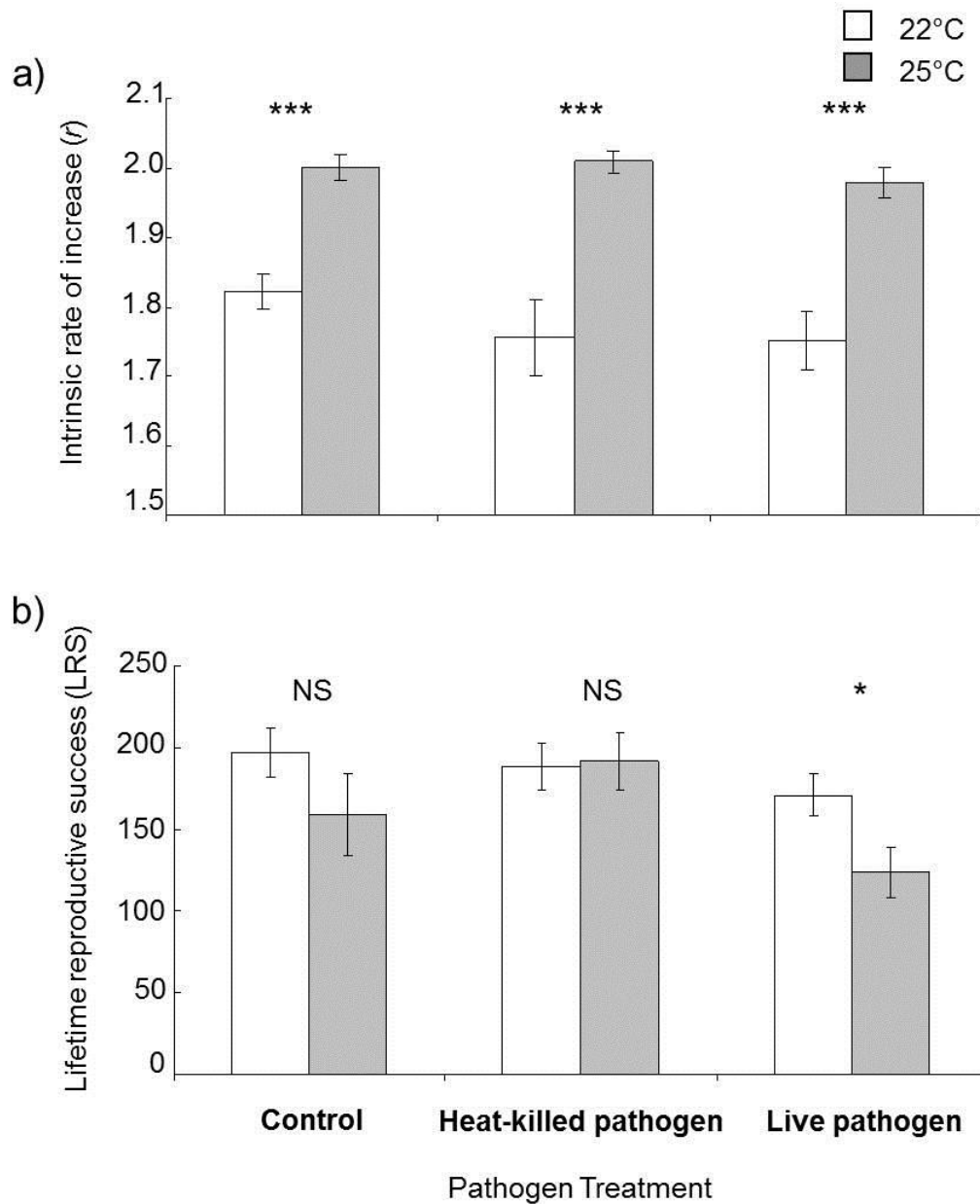


Figure 2: Fitness consequences of cold-seeking behaviour. **a)** Intrinsic rate of increase (r) across temperature and pathogen treatments. r was calculated using pupae produced at each collection interval for each block ($n = 30$). **b)** Lifetime reproductive success (LRS) across temperature and pathogen treatments. LRS was measured as the total number of eclosed pupae from the start of treatments until death for each female ($n = 244$). All data represent mean \pm SE. Statistical significance was established by a priori treatment contrasts specified in ANOVA (*, $P < 0.01$; ***, $P < 0.001$; NS, $P > 0.05$.)

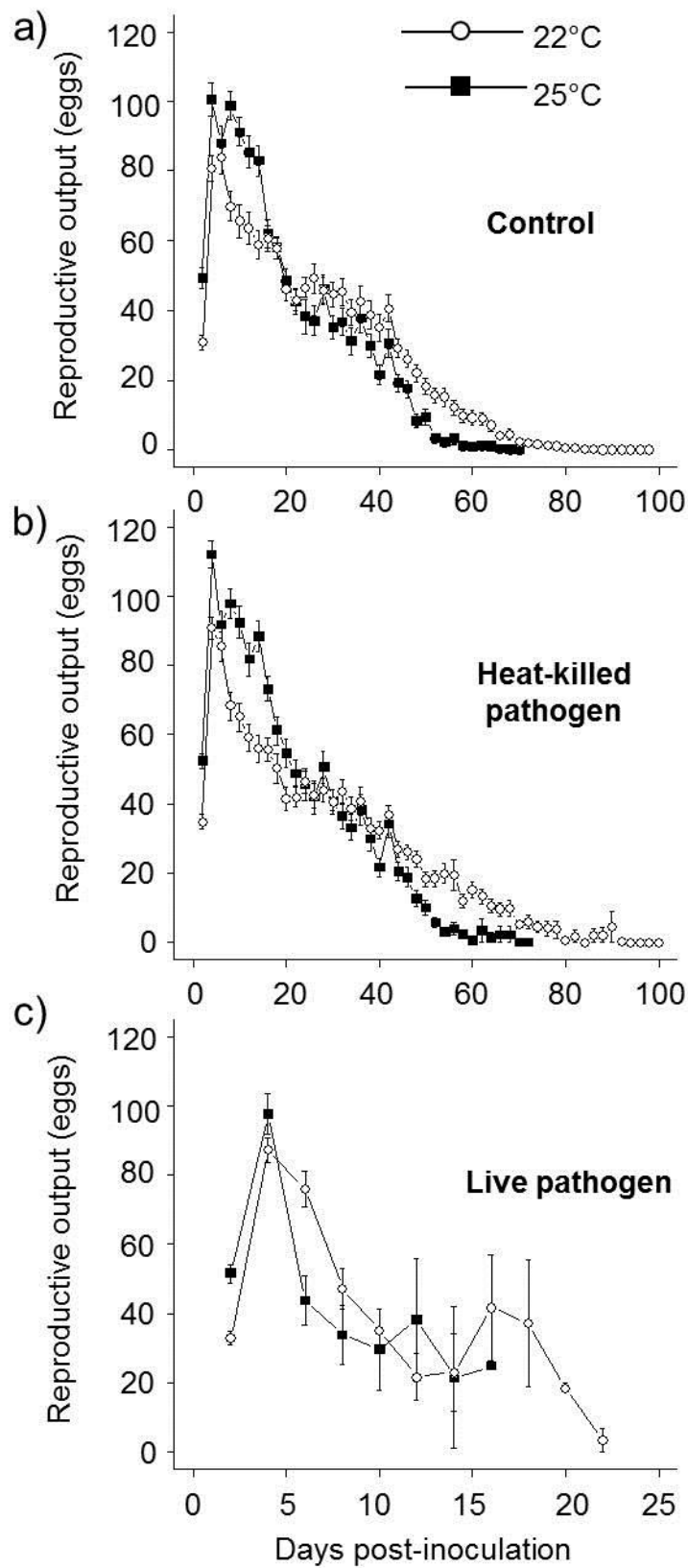


Figure 3: Age-specific fecundity patterns of *Drosophila* under different temperature and pathogen treatments. All data represent mean \pm SE.

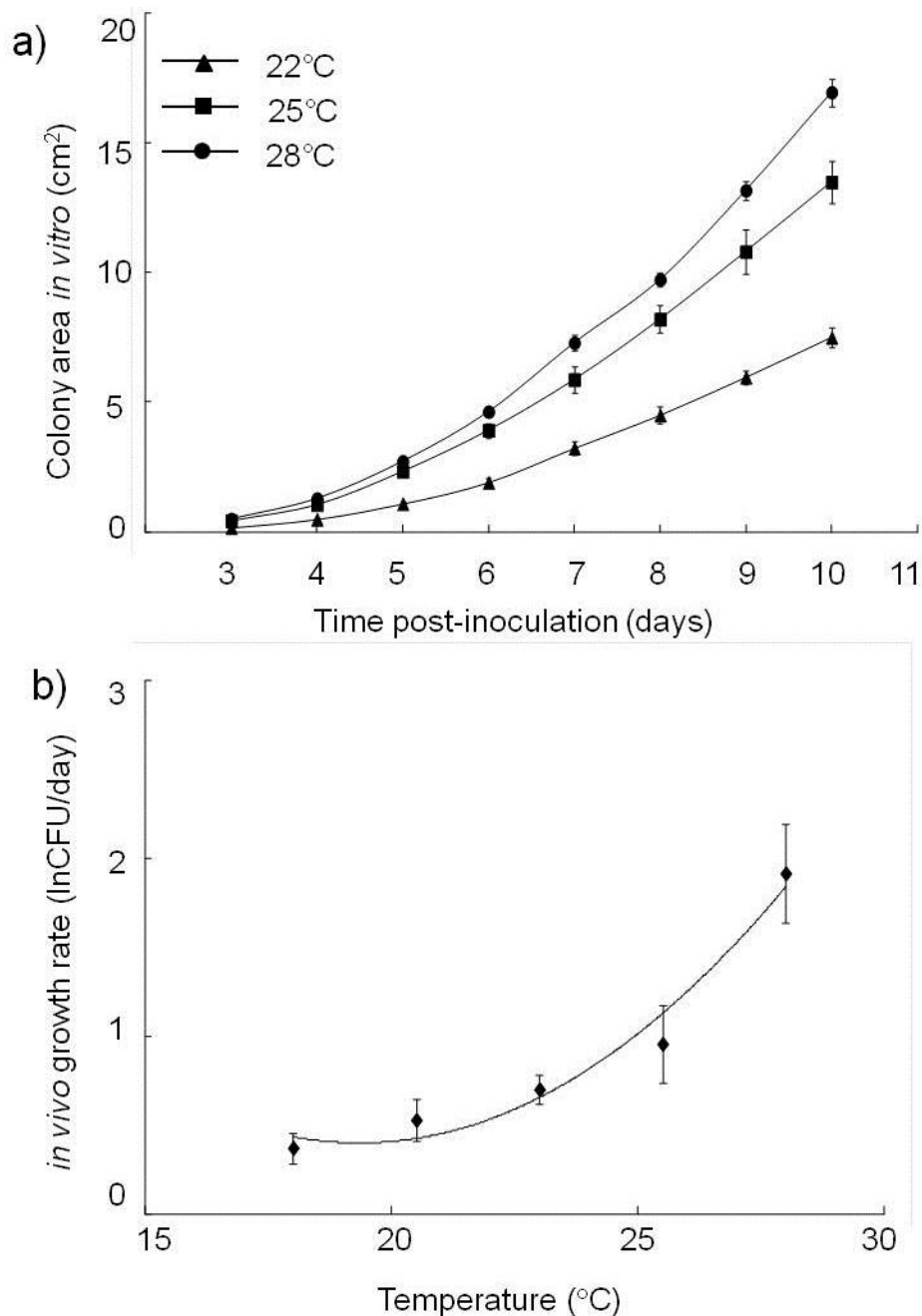


Figure 4: Moving to colder temperatures is detrimental to the fungal pathogen. a) *in vitro* colony growth of *Metarhizium robertsii* at 22, 25 and 28°C (Post-hoc Tukey's HSD tests revealed all pairwise comparisons were significant at $p < 0.0001$). b) *in vivo* rate of growth of fungal pathogen at 18, 20.5, 23, 25.5 and 28°C. Samples of live flies were taken at 3-4 day intervals and pathogen load was established by counting the number of colony forming units (CFUs) on replicate fungal media plates. The fitted line is least squares polynomial regression of natural-log transformed CFU counts between the first sampling point to peak CFU. All data represent mean \pm SE.

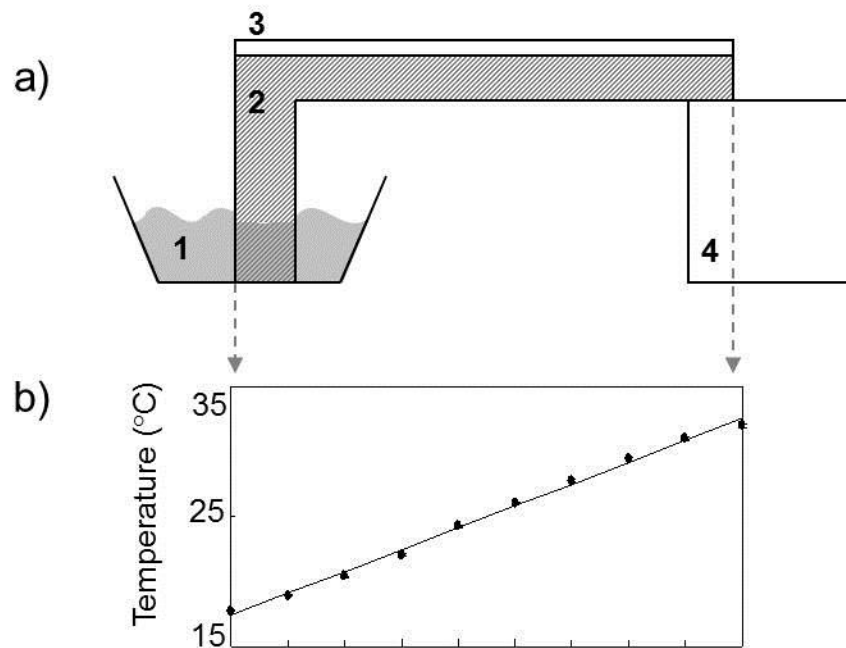
Supplementary Figures & Tables

Figure S1: Apparatus for measuring temperature preference of *Drosophila*. **a)** The experimental apparatus used to measure temperature preference. It consisted of a cold water bath (1), an 'L' shape aluminum block (270 x 180 x 30 mm) (2), a perspex lid (5mm in height) (3) and a hot plate (4). **b)** Measurements at fixed positions along the gradient indicate a temperature range of 16 to 32°C.

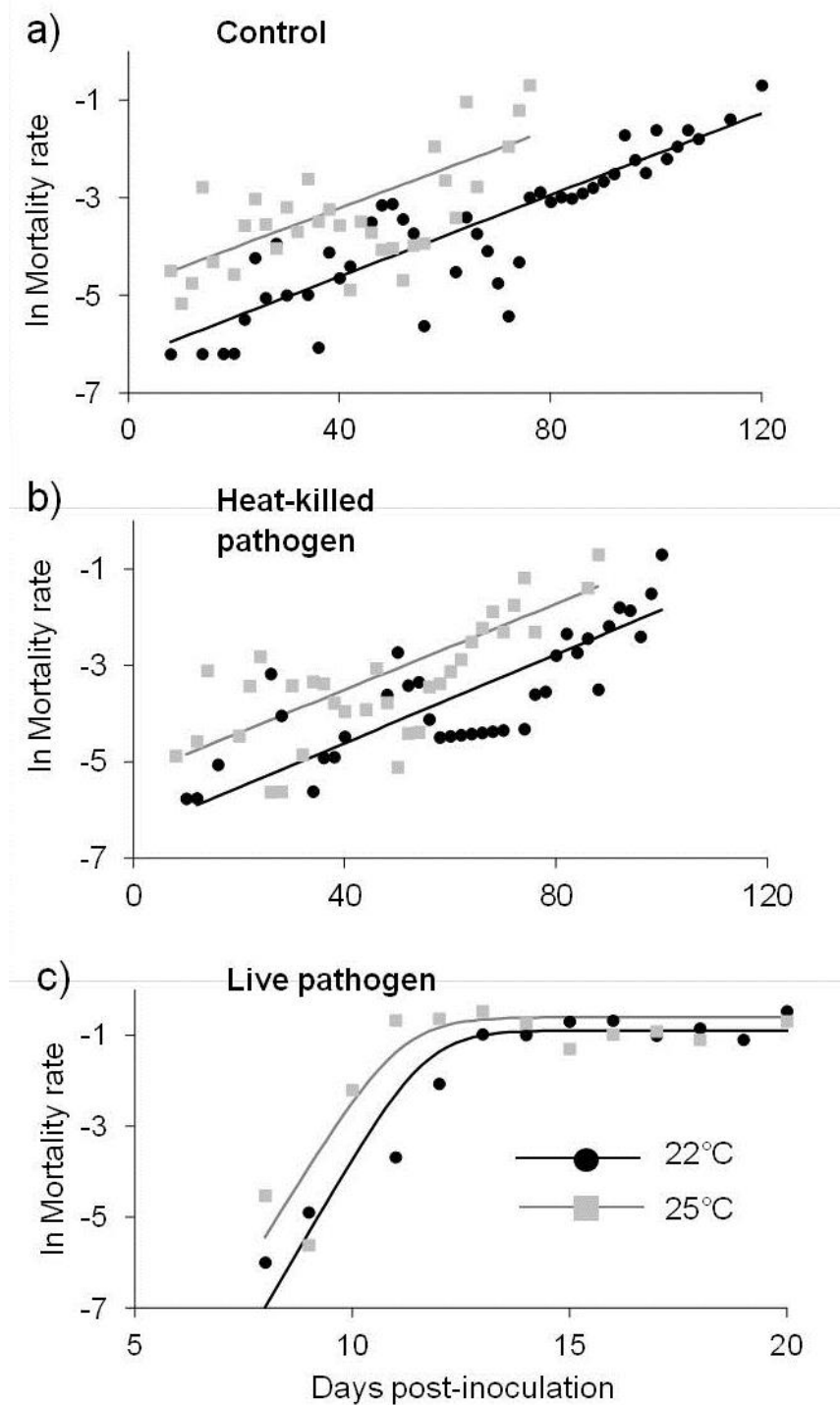


Figure S2: Temperature influences age-independent mortality while *Metarhizium* infection affects age-dependent mortality in *Drosophila*. **a)** Sham controls. **b)** Flies inoculated with heat-killed *M. robertsii* spores. **c)** Flies inoculated with *M. robertsii* spores. Data shown are natural log-transformed daily mortality rate. Fitted lines are Gompertz (a and b) and Logistic mortality models (c).

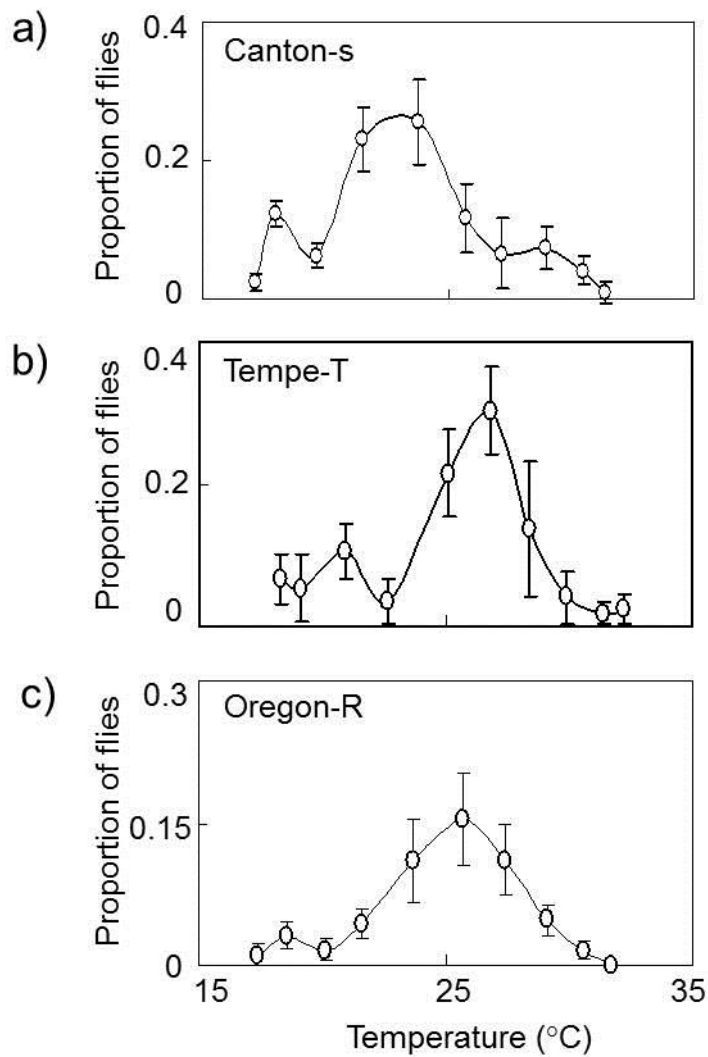


Figure S3: Temperature preference in three uninfected control laboratory strains of *Drosophila melanogaster*. **a)** *Canton-S* flies prefer mean temperature of $23.5 \pm 0.4^{\circ}\text{C}$ ($n = 77$). **b)** *Tempe-T* flies prefer mean temperature of $25.2 \pm 0.6^{\circ}\text{C}$ ($n = 37$). **c)** *Oregon-R* flies prefer mean temperature of $24.4 \pm 0.2^{\circ}\text{C}$ ($n = 220$). All data represent mean \pm SE.

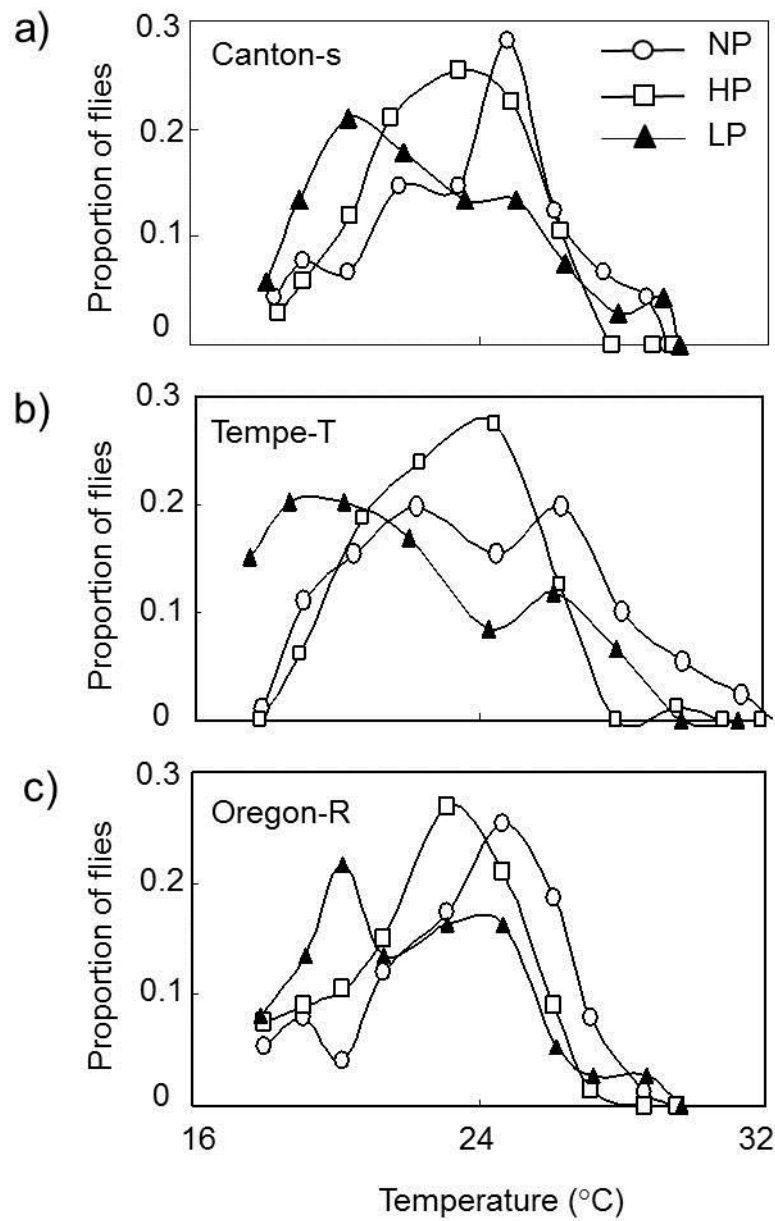


Figure S4: Temperature preference in *Drosophila melanogaster* exposed to live *Metarhizium* spores is shifted towards the cold. Temperature preference was assessed at 48 hours post-inoculation. **a)** *Canton-S* flies (n = 222). **b)** *Tempe-T* flies (n = 215). **c)** *Oregon-R* flies (n = 189).

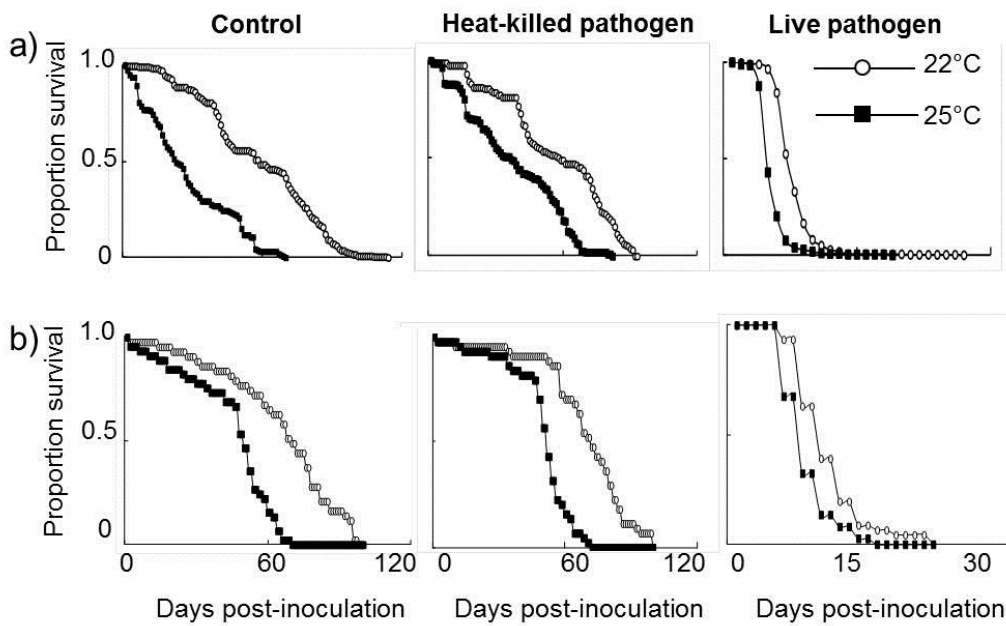


Figure S5: Cooler temperature enhances survival in all pathogen treatments across independent experiments. **a)** Survival curves for mixed sex fruit flies observed in large population cages used in the demographic experiment (n = 2088). **b)** Survival curves for female fruit flies observed in individual vials used in the life history assay (n=259).

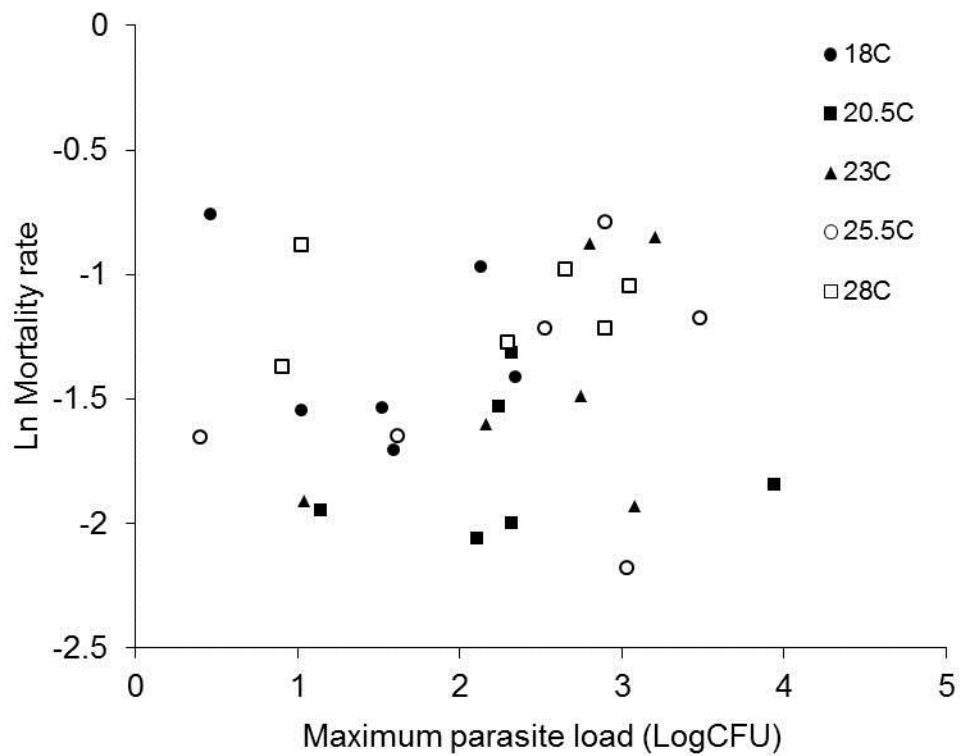


Figure S6: Temperature did not influence the relationship between pathogen load and host mortality (tolerance) of flies infected with *Metarhizium*. Plotted points are maximum CFU counts observed from each replicate cage population at each temperature regime. Mortality rates were estimated from the number of deaths from the same fly populations that provided pathogen load data.

Table S1: Analysis of variance terms and significance for the effect of pathogen and temperature on two fitness measures. Intrinsic rate of increase was estimated using each treatment combination within each block using the number of eclosed pupae produced at each collection interval ($n = 30$). Lifetime reproductive success was estimated as the total number of eclosed pupae over the entire lifetime for each female. Note that we excluded all females that produced fewer than 5 eclosed pupae prior to the analysis ($n = 233$).

Response variable	Effect	Test statistic and P value
Intrinsic rate of increase (r)	Pathogen	$F_{2,24} = 2.3, P = 0.118$
	Temperature	$F_{1,24} = 19, P = 0.001$
	Pathogen \times Temperature	$F_{2,24} = 1.0, P = 0.384$
Lifetime reproductive Success (LRS)	Pathogen	$F_{2,227} = 1.1, P = 0.341$
	Temperature	$F_{1,227} = 4.0, P = 0.047$
	Pathogen \times Temperature	$F_{2,227} = 1.9, P = 0.157$

Table S2: Mixed effects model terms and significance for overall age-specific fecundity models. Individual females were fitted as random intercepts. We excluded the first egg collection interval post inoculation (day 0-2) in the analysis because accurate model fitting was inhibited by the low levels of fecundity seen in these newly emerged flies.

Fixed effect	Test statistic and P value
Age	$F_{1,4923} = 218, P < 0.001$
Pathogen	$F_{2,248} = 24, P < 0.001$
Temperature	$F_{1,248} = 28, P < 0.001$
Age \times Pathogen	$F_{2,4923} = 145, P < 0.001$
Age \times Temperature	$F_{1,4923} = 77, P < 0.001$
Pathogen \times Temperature	$F_{2,248} = 0.4, P = 0.65$
A \times P \times T	$F_{2,4923} = 16, P < 0.001$

Table S3: Mixed effects model terms and significance for individual age-specific fecundity models. Individual pathogen treatments were fitted as separate mixed effects models. Mixed effects model was fitted to the entire data set after excluding the first egg collection interval post inoculation (day 0-2). Individual females are fitted as random intercepts.

Fixed effect	Test statistic and <i>P</i> value		
	Control	Heat-killed fungus	Live fungus
Age	$F_{1,2242} = 171$, $P < 0.001$	$F_{1,2444} = 264$, $P < 0.001$	$F_{1,237} = 107$, $P < 0.001$
Temperature	$F_{1,83} = 18$, $P < 0.001$	$F_{1,84} = 33$, $P < 0.001$	$F_{1,81} = 3.4$, $P = 0.069$
Age × Temperature	$F_{1,2242} = 53$, $P < 0.001$	$F_{1,2444} = 93$, $P < 0.001$	$F_{1,237} = 9.8$, $P = 0.002$

Table S4: Mixed effects model terms and significance for age-specific pathogen load. Model was fitted to log-transformed CFU data set after excluding measurements made on day 5 which only contained estimates for 28°C treatment. Individual cages were fitted as random intercepts.

Fixed effect	Test statistic and <i>P</i> value
Age	$F_{4,103} = 3.9$, $P = 0.007$
Temperature	$F_{1,28} = 0.2$, $P = 0.68$
Age × Temperature	$F_{4,103} = 3.4$, $P = 0.012$

Table S5: Mixed effects model terms and significance for the effect of temperature on the relationship between pathogen load and host mortality (tolerance). Model was fitted to natural-log transformed mortality rates. We used log-transformed CFU data set after excluding measurements made on day 5 which only contained estimates for 28°C treatment. Pathogen load was \log_{10} transformed. Age and temperature were fitted as categorical variables. Individual cages were fitted as random intercepts.

Fixed effect	Test statistic and <i>P</i> value
Pathogen load	$F_{1,98} = 4.6, P = 0.035$
Temperature	$F_{4,25} = 3.3, P = 0.026$
Age	$F_{5,98} = 107, P < 0.001$
Pathogen load x Temperature	$F_{4,98} = 1.0, P = 0.43$

Immune anticipation of mating in *Drosophila: Turandot M* promotes immunity against sexually transmitted fungal infections

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Abstract

Although it is well known that mating increases the risk of infection, we do not know how females mitigate the fitness costs of sexually transmitted infections (STIs). It has recently been shown that female fruit flies, *Drosophila melanogaster*, specifically upregulate two members of the *Turandot* family of immune and stress response genes, *TotM* and *TotC*, when they hear male courtship song. Here we use the *Gal4/UAS* RNAi gene knockdown system to test whether the expression of these genes provides fitness benefits for females infected with the entomopathogenic fungus, *Metarhizium robertsii* under sexual transmission. As a control, we also examined the immunity conferred by *Dif*, a central component of the *Toll* signalling pathway thought to provide immunity against fungal infections. We show that *TotM*, but not *TotC* or *Dif*, provides survival benefits to females following STIs, but not after direct topical infections. We also show that though the expression of *TotM* provides fecundity benefits for healthy females, it comes at a cost to their survival, which helps explain why *TotM* is not constitutively expressed. Together, these results show that the anticipatory expression of *TotM* promotes specific immunity against fungal STIs, and suggest that immune anticipation is more common than currently appreciated.

Running Title: *Drosophila* immunity against STIs

Keywords: Immune anticipation, sexually transmitted infections, ecological immunology, *Drosophila melanogaster*, *Metarhizium robertsii*, innate immunity.

Introduction

Mating is fraught with danger. In addition to the fitness costs associated with finding sexual partners, copulation and offspring production, mating increases the risk of acquiring sexually transmitted infections [1–3]. In insects, STIs are often both highly prevalent and pathogenic [3,4]. It is generally thought that they exert a selective pressure strong enough to influence the evolution of mating systems, life histories, sexual conflict and sexual behaviour [3,5]. Yet, we have a poor understanding of how they have shaped the immune system [6].

Females could mitigate the risks of acquiring STIs through immune anticipation of mating, the activation of immune responses before sexual congress and potential exposure to pathogens [7]. Pre-emptive immune activation is predicted to be more advantageous than a purely reactive response because it shortens the time delay of the immune response, and thereby maximises its efficiency ([7]; M.T. Siva-Jothy *et al.*, unpublished). We know that females upregulate a number of immunity-related genes in response to mating [8–12]. But, even the act of courtship might stimulate immune activation. If immune genes expressed during courtship represent immune anticipation of mating, then we would expect such responses to enhance immunity against STIs and to exhibit costs in some aspects of life history, because otherwise they would be constitutively expressed [13,14].

One way to address this possibility is to identify candidate immune genes associated with courtship, and perform infection and fitness assays in which the expression levels of the genes are manipulated. Recently, *Turandot C* and *Turandot M* (*TotC* & *TotM*), members of the *Turandot* family of immune and stress response genes, were shown to be upregulated in the heads of female *D. melanogaster* stimulated by male courtship songs independent of any physical encounter with males [15]. Of the two, *TotM* is likely to be the better candidate for anticipatory immunity against STIs, as it is poorly induced by non-immune related stress [16], but strongly induced by both fungal infections [16–18] and mating [9,19,20]. In addition, induction of *TotM* by natural fungal infection exhibits similar fold-change in expression to well-known antifungal antimicrobial peptides (AMPs) including *Drosomycin* and *Metchnikowin* [18]. Surprisingly, there is little evidence that courtship stimulates the upregulation of the canonical *Toll* and *Imd* pathway immune genes, such as *Dorsal-related immunity factor* (*Dif*), an NF- κ B- like factor

that regulates *Toll*-dependent immune responses thought to provide immunity specifically against gram-positive bacteria and fungi [15,21,22].

Previous efforts in establishing *Drosophila melanogaster* as a model laboratory system for studying insect STIs have focused on bacterial pathogens [23,24]. However, entomopathogenic fungi might be more appropriate. First, entomopathogenic fungi are widespread across diverse environments causing a large proportion of all known insect STIs and indeed, the majority of all insect diseases [3,25]. Second, because fungal spores cause infection through direct contact with the cuticle [26,27], they are amenable for comparisons between sexual and non-sexual horizontal transmission. Finally, studying the sexual transmission potential of entomopathogenic fungi in the laboratory have important implications for their application in the field as agents of biocontrol [28–30].

Here, we examine the hypothesis that *TotM* provides protection against sexually transmitted *Metarhizium robertsii*, a generalist soil-borne entomopathogenic fungus, which exhibits both sexual and non-sexual transmission in dipterans and has been used extensively in biocontrol [25,31,32]. Specifically, we test the predictions that 1) *Metarhizium* can be sexually transmitted in *Drosophila melanogaster*; that 2) expression of *TotM* helps to mitigate the cost of infections under sexual transmission, but not direct modes of transmission; and that 3) the expression of *TotM* has fitness costs in the absence of sexually transmitted *Metarhizium*. To address these questions, we use the Gal4/UAS RNAi targeted gene knockdown approach [33], in conjunction with large-scale demographic analysis, to estimate the immunity and fitness conferred by *TotM*, *TotC* and *Dif* under both STIs and high-dose direct topical infections (DTIs) of *M. robertsii*.

Material & Methods***Fly strains and fungal culture maintenance***

A wild-type Dahomey strain of *Drosophila melanogaster* (provided by Dr Stuart Wigby, University of Oxford) was kept in large population cages (1m³) with overlapping generations for two years prior to the start of the experiments. RNAi strains were obtained from Vienna *Drosophila* RNAi Center (UAS-*TotM*-IR, transformant ID 106727; UAS-*TotC*-IR, transformant ID 106379; UAS-*Dif*-IR, transformant ID 30579, see [31]). We used the non-tissue specific Act5C promoter [34] to drive ubiquitous expression of Gal4 and UAS constructs (Act5C-Gal4/CyO, Bloomington Stock Centre stock number 4414). We crossed Act5C-Gal4/CyO females with males carrying one of the UAS constructs to generate the active knockdown genotypes (Act5C-Gal4/UAS-*TotM*-IR; Act5C-Gal4/UAS-*TotC*-IR; Act5C-Gal4/UAS-*Dif*-IR). As a control for the presence of the UAS transgene, we crossed w1118 wild-type females (the genetic background for all RNAi lines, obtained from Bloomington Stock Centre) with males carrying one of the UAS constructs (UAS-*TotM*-IR/+; UAS-*TotC*-IR/+; UAS-*Dif*-IR/+). As a control for the presence of the Gal4 driver, we crossed Act5C-Gal4/CyO females with w1118 males (Act5C-Gal4/+). The effectiveness of RNAi knockdowns of *TotM* and *TotC* was confirmed by semi-quantitative PCR [35]. All experimental animals were maintained at 25°C with 12:12 light-dark cycle in standard *Drosophila* vials at low densities (~50 flies/vial) for at least two generations prior to the start of experiments. We used an oatmeal-molasses-agar media with added live baker's yeast and an antifungal agent (Nipagin), which inhibited the growth of naturally-occurring saprophytic fungi. All experimental flies used were collected as virgins over a period of 24 hours.

Metarhizium robertsii (isolate 2575, previously known as *Metarhizium anisopliae* strain ME1) was obtained from the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF, United States Department of Agriculture). We inoculated quarter-strength sabouraud dextrose agar (SDA) with *M. robertsii* conidia (asexual fungal spores) and incubated the plates at 28°C for four weeks before storing at 4°C for up to three months. Conidia were collected by scraping the surface of the sporulating culture with an inoculating loop.

Sexual transmission of fungal pathogen

We assessed the transmission potential of *M. robertsii* by exposing healthy Dahomey females to males that had been topically inoculated with the fungus. At adult age day 4, groups of ten virgin males were topically inoculated with 6 mg of conidia without CO₂ anaesthesia by shaking in a 250ml conical flask for 20 seconds. Inoculated flies were held in temporary holding vials for 24 hours, ensuring that they had opportunities to groom themselves, which has previously been shown to be effective at removing fine dust particles [36]. At adult age day 5, each infected male fly was introduced into a new vial containing 10 uninfected virgin females of the same age and removed after 24 hours. The logic of giving males time to groom and subsequently using a fresh vial was to allow male to adopt a more natural behaviour [32], and to minimise the probability of females contracting infection from conidia that had been dislodged during grooming. We then transferred and held treated females in individual vials for a further 24 hours to allow egg-laying. The presence of larvae four days after oviposition indicated that the female had mated with an infected male. We assessed the infection status of females by the presence of *Metarhizium*-like fungal growth on cadavers. Flies were briefly immersed in 70% ethanol before being gently crushed and placed in Petri dishes on moistened filter paper at the end of the egg-laying period. After an incubation period of 5 days at 28°C, we examined all cadavers for signs of *Metarhizium*-like fungal growth (either hyphae or conidia) with a low-power dissection microscope. Because high levels of horizontal transmission of conidia between infected and naïve flies due to non-sexual contact could confound our interpretation, we also assessed the potential for non-sexual horizontal transmission of *M. robertsii* using the same procedures described above by exposing naïve males and females to infected flies of the same sex.

Survival assays under DTI and STI

We assessed the effects of gene knockdowns on survival under high-dose direct topical infections (DTI) and sexual transmission (STI) using adult flies for all genotypes. For DTI, at adult age day 7, we infected groups of approximately 300 mixed sex flies of each genotype with 20mg of conidia, or kept as uninfected control, following the protocol described previously. Inoculated flies were held in temporary holding vials for 30 minutes before being transferred to demography cages (10×15cm). For STI, we first inoculated 6-

day old w1118 males in groups of 20 with 12mg of conidia, and then transferred 20 infected or control males with 20 uninfected females to demography cages at adult age day 7. As infected males in STI treatment suffered much greater mortalities than control males, we restored the original complement of 20 infected males by adding freshly infected w1118 males at day 12 and 24 post-inoculation. For both DTI and STI, we removed and recorded dead flies daily until day 9 post-inoculation and every two days thereafter. We also tracked the changes in pathogen loads in the first 24 hours following DTI by sampling inoculated Dahomey wild-type flies at three time points post-inoculation (0 hour, 2.5 hours and 24 hours; n=9). Sampled flies were individually homogenised in 200µl of 0.04% Tween80[®], diluted by a factor of 10³ and spread onto standard SDA plates. Pathogen loads were assessed by counting the numbers of colony forming units (CFUs) following incubation at 28°C for 24 hours.

Fecundity assay under STI

We assessed the effects of gene knockdowns on survival and fecundity of females exposed to fungus-infected males using flies from the same cohort collected for survival assays. In the fecundity assay, we first infected two-day old w1118 wild-type adult males (the genetic background of our RNAi strains). At 24 hour-post inoculation, infected or uninfected control males were transferred to individual vials containing a single uninfected virgin female for each genotype. The mating pairs were assigned positions in randomised blocks and transferred to new vials after 24 hours, and thereafter every two days until day 9 (n=55/treatment/genotype). Used food vials were frozen 18 days after collection and the numbers of hatched pupae were counted giving a combined measure of fecundity and larval viability. We assessed the proportion of females that became infected through mating with infected males by sampling all surviving females at the end of day 9 post-inoculation (96.8%, 701/724) and checking for signs of *Metarhizium*-like fungal growth after incubation at 28°C for up to two months.

Statistical analysis

All statistical analyses were performed with R version 2.15 [37]. We assessed the contribution of mating to the transmission of STIs by comparing the proportions of flies that displayed *Metarhizium*-like fungal growth for mated females, and those that were

kept with infected males but remained virgin using chi-square tests with continuity correction. We used student's t test on CFUs to directly compare pathogen loads immediately after inoculation and after 24 hours.

Cox proportional hazard regressions were used to analyse all survival data. The full model (including all genotypes) contained age at death and censoring information as the response variables; genotype, infection treatment and their interaction were included as predictor variables. A separate Cox regression was performed for each gene of interest that only included the relevant knockdown and control genotypes (e.g. for *TotM*, the data included these genotypes: Act5C-Gal4/UAS-*TotM*-IR, Act5C-Gal4/+ and +/UAS-*TotM*-IR). For each gene of interest, we first extracted the hazard ratios (the fold-increase in risk of death in infected animals relative to uninfected controls) for the knockdown genotype and its combined control genotype (by pooling raw survival data of the relevant control genotypes) from the Cox models. Because the mortality rate in the DTI treatment is substantially higher than in the STI treatment, it is difficult to directly compare the effect of immune gene knockdowns in the two treatments. To overcome this problem, we calculated normalised hazard ratios by dividing the hazard ratios of each knockdown by its associated combined control genotype. Unlike simple metrics of lifespan, this measure describes the effect of each gene knockdown on immunity after accounting for its genetic background, which allows us to directly compare the immune properties conferred by genes under STIs and DTIs, despite great differences in effect size. We assessed the survival cost of gene expression in the absence of infections by comparing the hazard ratios of each gene knockdown relative to its combined control genotype under uninfected control conditions.

We used mixed effects models to assess the effects of genotype and infection on fecundity across time. The full model included the number of hatched pupae produced at each time point as the response variable; genotype, treatment, time and all associated two-way interactions as fixed effects (three-way interaction was non-significant when fitted and thus dropped from the full model), and individual females as random effect (intercepts). We also included the age at death of male partners as a covariate in the full model to account for the possibility that females might have lower fecundity under STI simply due to a lack of remating opportunities as infected males die at earlier ages than uninfected controls. Female fecundity in the first 24 hours was excluded from the model

as the fecundity was much lower than at other time points and previous experiments suggested minimal *in vivo* fungal growth in this period (Hunt *et al.* unpublished). We assessed the fecundity cost of gene expression in the absence of infections by comparing the mean total pupae productions of the gene knockdown (day 0-9 post treatment) and the combined control genotype using one-way analysis of variance.

Results

Sexual transmission of fungal pathogen

We found that *Metarhizium robertsii* can be sexually transmitted in the fruit fly, with approximately one in five (55/263) naïve females displaying *Metarhizium*-like fungal growth on their cadavers after being placed with a topically infected male for 24 hours (Figure 1a & b). Further analysis showed that fungal transmission was driven primarily by mating, as the proportion of cadavers with fungal growth was higher in gravid females than infertile females ($\chi^2_1 = 9.0$, $P = 0.003$; Figure 1c). The dose received by females was likely to be low as the pathogen load of the topically infected males was only ~5,000 CFU, which had declined by grooming from the initial load of ~20,000 CFUs ($t = 7.7$, $P = 0.006$; Supplementary Figure 1). Finally, we also found that *Metarhizium* could be transmitted among same sex flies (7/277 for male-to-male transmission and 7/266 for female-to-female transmission; Supplementary Figure 2). Nevertheless, naïve flies were much more likely to be infected through sexual transmission than non-sexual transmission, 20.9% vs. 2.6%, respectively.

Effects of STI and DTI on survival across RNAi strains

We found that *TotM* promotes immunity against *Metarhizium* when it is sexually transmitted (STI), but not when it is applied as a direct topical infection (DTI). The effect of STIs on the hazard ratio, which estimates the risk of death in infected treatments relative to control treatments, was highly dependent on the host genotype (Overall: Genotype \times Treatment, $\chi^2_6 = 26$, $P = 0.001$; Figure 2a). Specifically, *TotM* knockdown flies (Act5C-Gal4/UAS-*TotM*-IR) were susceptible to STIs, but there was no evidence of susceptibility in either of +/Act5C-Gal4 or +/UAS-*TotM*-IR control genotypes (Genotype \times Treatment; $\chi^2_2 = 16$, $P = 0.001$; Figure 2a). In contrast, there was no difference in susceptibility to STIs among *Dif* knockdown flies (Act5C-Gal4/UAS-*Dif*-IR) and its associated control genotypes +/Act5C-Gal4 and +/UAS-*Dif*-IR (Genotype \times Treatment, $\chi^2_2 = 0.1$, $P = 0.98$). Surprisingly, *TotC* knockdown flies (Act5C-Gal4/UAS-*TotC*-IR) had slightly higher survival post-exposure than both of control +/Act5C-Gal4 and +/UAS-*TotC*-IR genotype flies (Genotype \times Treatment, $\chi^2_2 = 9.1$, $P = 0.011$; Figure 2a).

We found different patterns under direct topical infection. While DTIs generally caused very rapid mortalities such that 95% of flies died within 9 days, some genotypes were much more susceptible (Overall: Genotype \times Treatment, $\chi^2_6 = 751$, $P < 0.001$; Figure 2b). As expected [22], *Dif* knockdown (Act5C-Gal4/UAS-*Dif*-IR) females were significantly more susceptible to DTIs than either of its control genotypes (Genotype \times Treatment, $\chi^2_2 = 545$, $P < 0.001$; Figure 2b). However, neither *TotM* nor *TotC* knockdown was more susceptible to DTIs than their respective control genotypes (Figure 2b). Interestingly, although the hazard ratio of the *Dif* knockdown line under DTI was more than 16 times higher than that of *TotM* knockdown under STI (46.2 ± 6.2 vs 2.8 ± 0.6), their hazard ratios were comparable after they were normalised to account for the susceptibility of their control genotypes (2.4 ± 0.4 vs 2.7 ± 0.7 ; Figure 2c).

Effect of STI on fecundity across RNAi strains

Sexually transmitted *Metarhizium* infections resulted in reproductive costs for female flies. Exposure to topically infected male partners initially had little impact on female reproduction, but over time, female fecundity in the infected treatment declined relative to uninfected controls (Treatment \times Time, $F_{1,2030} = 30$, $P < 0.001$; Supplementary Figure 3). This pattern was consistent in all lines as there was no evidence that *TotM* or indeed any gene knockdown strain suffered greater fecundity reduction than their control genotypes (Treatment \times Genotype, $F_{6,705} = 1.5$, $P = 0.19$). The reduction in female fecundity under STIs could not be explained by a lack of remating opportunities due to increased mortalities of infected male partners, because male longevity did not significantly contribute to female fecundity over the course of the experiment ($F_{1,705} = 3.5$, $P = 0.062$). In addition, while the cadavers of females that had been exposed to infected males were more likely to exhibit *Metarhizium*-like fungal growth than those exposed to control males ($\chi^2_1 = 5.7$, $P = 0.017$), there was no evidence that the RNAi knockdown genotypes influenced the probability of fungal growth ($\chi^2_1 = 0.0$, $P = 0.97$; Supplementary Figure 4).

Effect of immune gene expression on survival and fecundity in uninfected flies

We found that the expression of *TotM* and *Dif*, but not *TotC*, results in survival costs for uninfected females. Both *TotM* and *Dif* knockdown flies (Act5C-Gal4/UAS-*TotM*-IR and

Act5C-Gal4/UAS-*Dif*-IR), but not *TotC* knockdown flies (Act5C-Gal4/UAS-*TotC*-IR), showed enhanced survival relative to their control genotypes (*TotM*: $\chi^2_1 = 8.6$, $P = 0.003$; *Dif*: $\chi^2_1 = 27$, $P < 0.001$; *TotC*: $\chi^2_1 = 0.9$, $P = 0.35$; Figure 3a). In contrast, we found evidence for reproductive benefits of *TotM* and *TotC* expression, but reproductive costs of *Dif* expression. Both *TotM* and *TotC* knockdown females had lower total reproduction than their respective controls, while *Dif* knockdown females were more fecund than its control genotypes (*TotM*: $F_{1,135} = 45$, $P < 0.001$; *TotC*: $F_{1,127} = 7.6$, $P = 0.007$; *Dif*: $F_{1,129} = 6.3$, $P = 0.014$; Figure 3b).

Discussion

Mechanisms of insect immunity are known to be pathogen-specific [38,39]. However, the extent to which insects use ecological cues to inform which responses to mount is not known. Our study shows that a gene that is upregulated in anticipation of mating provides protection against sexually transmitted *Metarhizium* infections. This finding is important because it illuminates the molecular mechanisms as well as the life history costs and benefits which underpin immunity against STIs. In combination with previous results [15], our results imply that fruit flies demonstrate immune anticipation of mating and that immune anticipation could be a general mechanism for achieving immune specificity.

A Turandot gene that enhances immunity against STIs

Hundreds of *Drosophila* genes, including *TotM*, have been identified on the basis of elevated expression following immune challenges, but the functional consequences of these genes are rarely established [16–18]. This is a problem because gene expression does not necessarily translate into immunity against live pathogens [40–42]. We show that *TotM* confers protection to fungal STIs, and its effects are similar in magnitude to that conferred by *Dif* to fungal DTIs.

The mechanisms through which *TotM* enhances immunity are currently unknown. All protein products encoded by the *Turandot* gene family are thought to be actively produced in the *Drosophila* fat bodies and secreted into the haemolymph, where they are hypothesised to act as protein chaperones or as signalling molecules [16,43]. Though direct tests are needed, it seems unlikely that *TotM* possesses direct antimicrobial activities similar to known antifungal AMPs such as *Drosomycin* and *Metchnikowin*; since over-expression of another *Turandot* gene, *TotA* does not provide increased protection against gram-negative bacterial infections [43,44]. Instead, *TotM* might help the fly to tolerate persistent fungal infections by mitigating the negative effects of the infection without actively suppressing pathogen growth [45–47]. Consistent with a role in enhancing tolerance, not resistance, we found that fungi were as likely to emerge from the control genotype flies as they were from *TotM* knockdown flies.

Mode of transmission and immunity

Fruit flies have a remarkable ability to mount immune responses which are specific to the pathogens they encounter [38,39]. Our work shows that the efficacies of their immune responses are also specific to the mode of infection transmission. STIs differ from other modes of transmission in that they tend to cause chronic low level infections, which do not result in rapid septicaemia and increased host mortality – consequences typically associated with acute immune challenges [2]. The lower initial inoculum in our STI treatment is evidenced by the proportion of flies that exhibit fungal growth on female cadavers (5-25% for STIs and 80-95% for DTIs; Hunt *et al.*, submitted); and the increased grooming activities we observed in the DTI treatment, which efficiently reduced pathogen load (this study; [36]). Consistent with the differences in pathogen dose between the two infection treatments, we found that sexually transmitted *Metarhizium* infections cause weak, though significant, fitness costs for females, and that the expression of *TotM*, but not *Dif*, ameliorates the survival costs associated with STIs. In contrast, we found that direct topical *Metarhizium* infections cause substantial fitness costs for females, and that the expression of *Dif*, but not *TotM*, helps ameliorate those survival costs. Taken together, these findings show fruit flies have a specific mechanism for immunity against low-dose STIs and against high-dose DTIs, even for the same pathogen.

It is important to acknowledge that though we have established a role for *TotM* in immunity against low-dose STIs, we do not know whether *TotM* confers immunity against STIs *per se*, or to low-dose infections more generally. We cannot dismiss the possibility that high fungal doses overwhelmed the fine-tuned protective effects provided by *TotM* or that low fungal doses masked the susceptibility of the *Dif* knockdown. Similarly, the choice of diet could confound our results, as the fecundity benefits of *TotM* and *TotC* expression might have resulted from the *ad libitum* access to dietary yeast in this study [48]. Another potential problem is that genetic constructs such as the Act5C driver and UAS element may have pleiotropic effects on the life history of the fly, which could confound direct comparisons with the knockdown genotype. However, these problems are unlikely to influence our interpretations. The response to topical fungal infection in our *Dif* knockdowns was similar to that of the classic *Dif* knockout mutant [22]. Because our experiments were conducted under the same dietary conditions and because our analysis included normalizations to control genotypes, we can confidently attribute the survival

reduction in *TotM* knockdown to the effect of gene expression, rather than potential confounding factors such as diet, genetic pleiotropy, or the general frailty of immune gene knockdown lines [49]. Regardless of how they confer immunity, our findings provide clear evidence that *TotM* and *Dif* are specific for different modes of fungal transmission, and that their expressions have different life history consequences for the host.

It is important to stress that we are not arguing that *M. robertsii* is predominantly transmitted sexually or claiming that it is transmitted internally during copulation. Given the proclivity of *Metarhizium* for topical transmission, we would expect there to be some non-sexual transmission, even in our STI treatments. *Drosophila* tend to aggregate on food sources, which could have increased contacts and fungal transmission in the present study [50]. However, non-sexual transmission is unlikely to be substantial enough to change the interpretation of the data. First, males had been given 24 hours for grooming and were subsequently placed in fresh vials, which reduced the risk of females indirectly picking up dislodged spores. Second, we found that females who mated with infected males were more likely to be infected than those that did not. And, finally, in independent experiments, infection success was substantially lower in same-sex transmission trials than in trials involving sexual transmission (21% vs. 3%). Thus, although we documented that the fungus can be transmitted non-sexually, sexual transmission is primarily responsible for the observed infections in our STI treatments.

The cost of immune expression

Though many studies have documented the costs of immunity [14,48,51], the molecular and physiological basis of such costs are often poorly understood [13]. We found that under uninfected control conditions *Dif* is generally deleterious in the absence of infections. The expression of *Dif* entails both significant survival and fecundity costs, which is also supported by a previous study of *Dif* knockout mutant [49]. The costs of *Dif* expression are likely to arise from its control of AMP induction through the *Toll* pathway [22], though *Dif* might also function in other non-immunity related processes [21]. These strong fitness costs could help to explain why *Dif* only appears to be modestly induced by direct topical fungal infections [17], and why it was not upregulated in females in response to male courtship songs (at least in their heads) [15].

In contrast, our findings for the *Turandot* genes are only partially consistent with the predicted costs of immune gene expression. We found that *TotM* has an antagonistic pleiotropic influence on the life history of the fly: though it is costly for survival, expression of *TotM* also substantially enhances female fecundity. In addition, while there was no evidence that *TotC* conferred immunity against *Metarhizium*, it did not contribute to survival cost and even enhanced female fecundity. However, unlike *Dif*, there is evidence that *TotC* and *TotM* play additional roles in reproduction. In particular, *TotC* and *TotM* are upregulated in response to exposure to male accessory gland proteins [8–12]. Perhaps *TotM* could mediate the trade-off between late-age survival and early-age reproduction, a key component of fitness in populations with fluctuating growth rates [52]. Thus, though we cannot easily tease apart the cost of expression from the additional roles played by *TotM*, the fact that its expression induces survival costs indicates that *TotM* has a long-term detrimental effect, which is an important facet of the explanation for why it is not constitutively expressed. Interestingly, *TotM* and *TotC* appear to evolve more rapidly than *Dif* [53], suggesting that they have experienced divergent or relaxed selection, perhaps as a consequence of their lower cost of expression [14,48,54].

Mating and immune anticipation in insects

Mating is frequently associated with heightened risk of contracting both ‘pure’ STIs and other opportunistic infections [3,55–57]. Such threats could be countered by upregulating immunity-related genes in post-mating [8–12]. However, because of the full deployment of immune responses can often take a considerable amount of time [58,59], selection is expected to favour immune anticipation of mating [7]. Though there have been few well-documented cases, immune anticipation is likely to be far more common than currently appreciated. Our study supports the hypothesis that female fruit flies can mitigate the risk of contracting sexually transmitted fungal infections during mating by pre-emptively upregulating *TotM* [15]. More generally, there are many other biological scenarios associated with elevated disease risk for which we would expect immune anticipation to be advantageous, such as feeding (as has been documented in bed bugs, M.T.Siva-Jothy *et al.*, unpublished) and crowding of conspecifics [60–62]. A particularly tantalising possibility is that the control of many immune genes including *TotM* [63], by circadian clock genes might reflect ‘anticipation’ of predictable fluctuations of disease risk over the

course of 24 hours. Thus, the courtship-induced, pre-emptive upregulation of *TotM* might be representative of a general pattern of immune anticipation in insects, underlining the intimate link between brain, behaviour and immunity [64,65].

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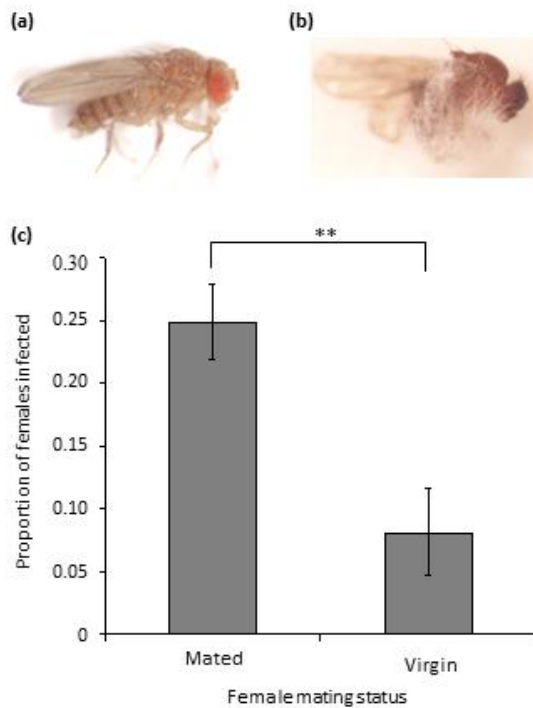
Figures & Tables

Figure 1: *Metarhizium robertsii* can be horizontally transmitted in *Drosophila melanogaster* as a result of mating. **(a)** a female *Drosophila* covered in *Metarhizium* conidia immediately after direct topical infection; **(b)** growing hyphae of *Metarhizium* emerging from infected fly cadaver; **(c)** when kept in a cage with a *Metarhizium*-inoculated male, females that had been inseminated were much more likely to acquire conidia than those that remained infertile.

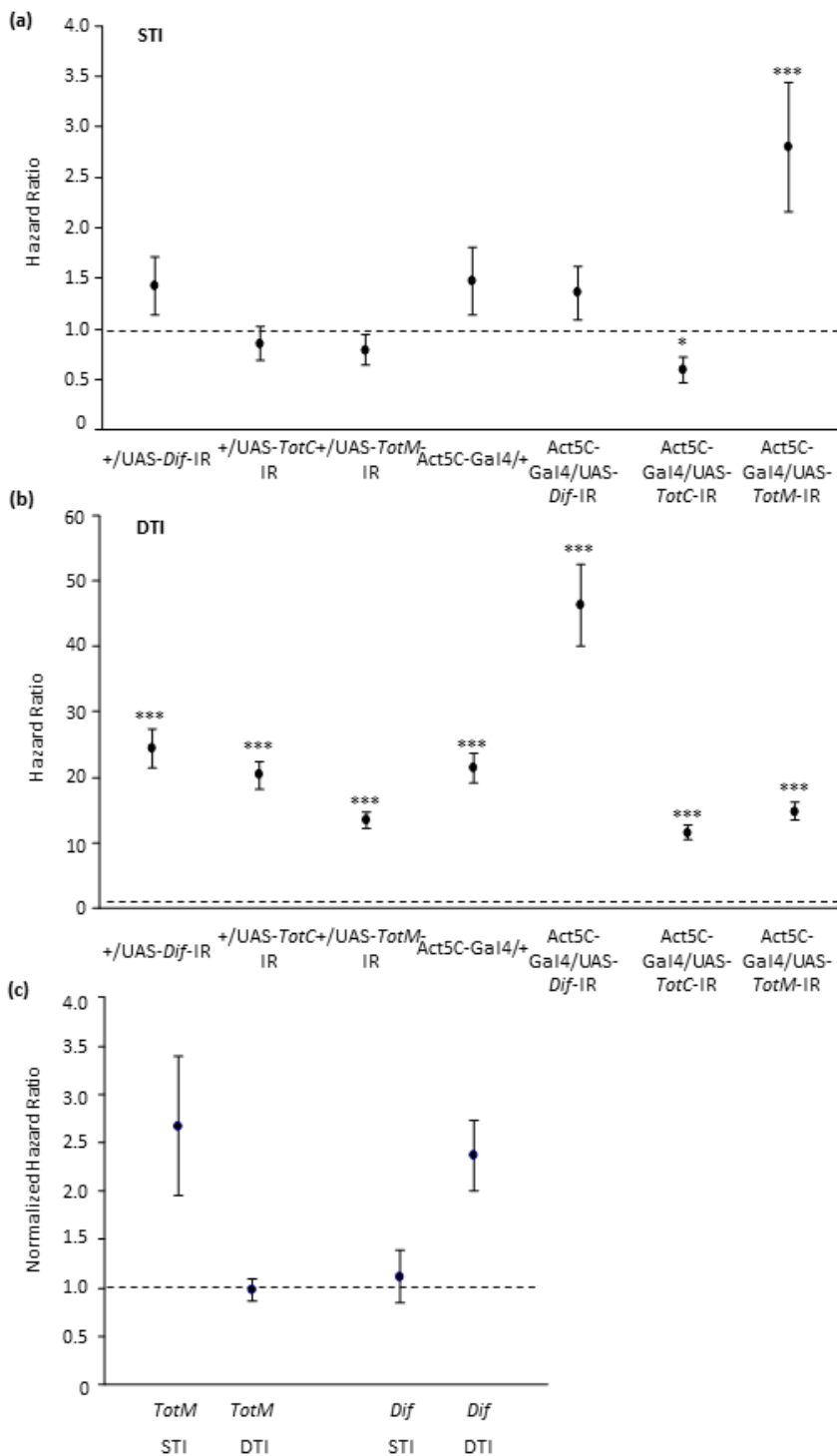


Figure 2: *TotM* is required for enhanced survival under sexually transmitted infection (STI), but not under direct topical infection (DTI). **(a)** Cox proportional hazard ratios of STI relative to uninfected controls; **(b)** Cox proportional hazard ratios of DTI relative to uninfected controls; **(c)** the susceptibility of *TotM* and *Dif* under both STI and DTI after normalization for differences in the influence of mode of infection on hazard of the control genotypes. Dotted lines indicate hazard ratio of 1, which indicate both infected

and uninfected controls had the same risk of death. * indicates the level of statistical significance of hazard ratios (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

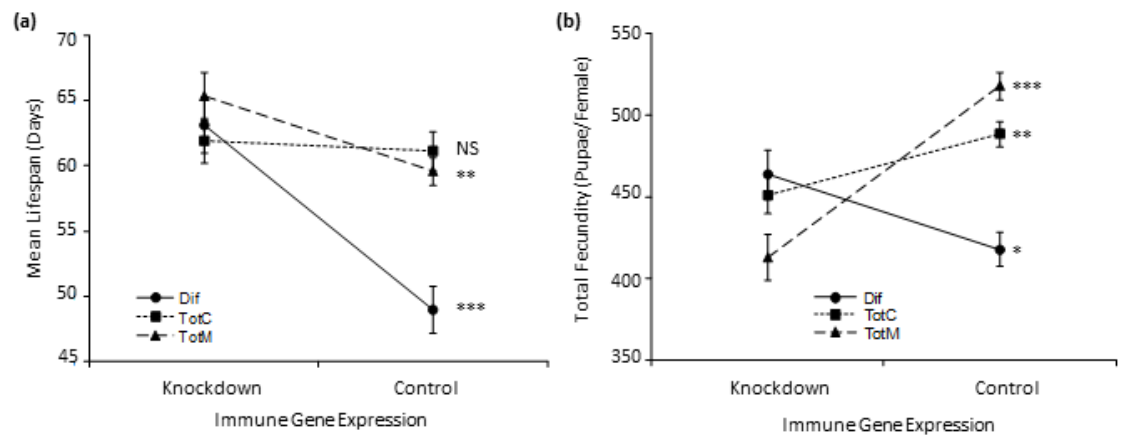


Figure 3: The costs of immune gene expression in the absence of infections. **(a)** survival costs as measured by mean lifespan; **(b)** fecundity costs as measured by total number of hatched pupae in the first 9 days post infection. * indicates level of statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). For survival costs, statistical significance was based on Cox proportional hazard regression of the survival curves of knockdown and its combined control. For fecundity costs, statistical significance was based one-way ANOVAs.

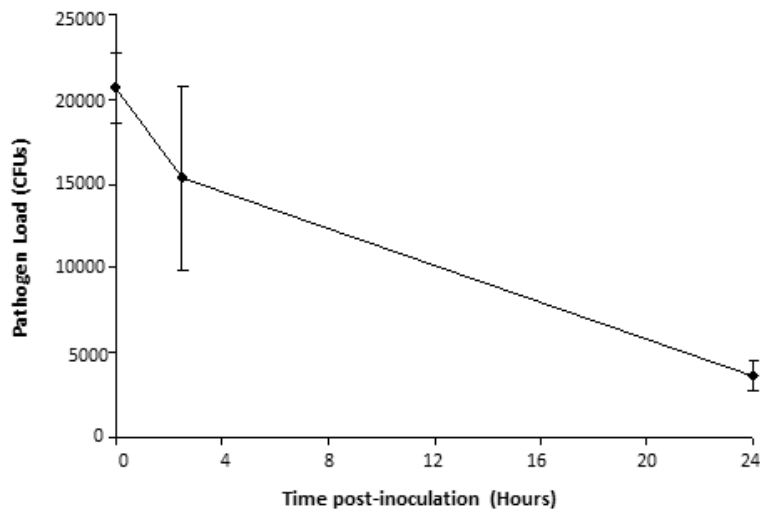
Supplementary Figures & Tables

Figure S1: Fungal conidia load declines rapidly in the first 24 hours post inoculation ($n = 3$ at each time point)

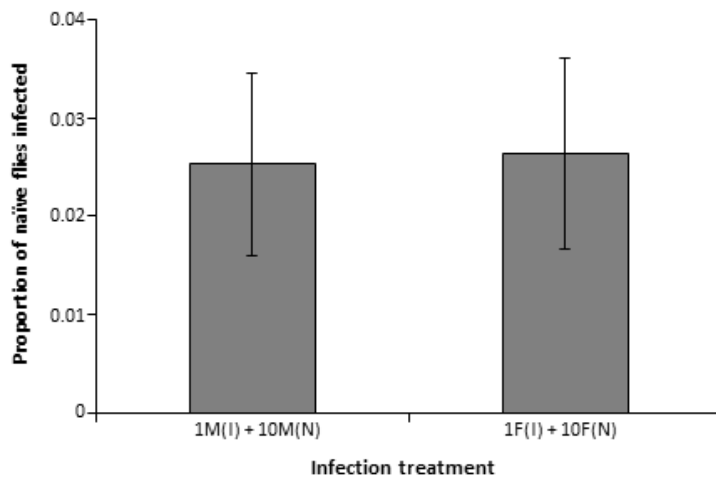


Figure S2: Non-sexual transmission of *Metarhizium robertsii* between fruit flies. M(I), infected male; M(N), naïve male; F(I), infected female; F(N), naïve female. Sample size of naïve flies: 1M(I) + 10M(N), $n = 284$; 1F(I) + 10F(N), $n = 273$

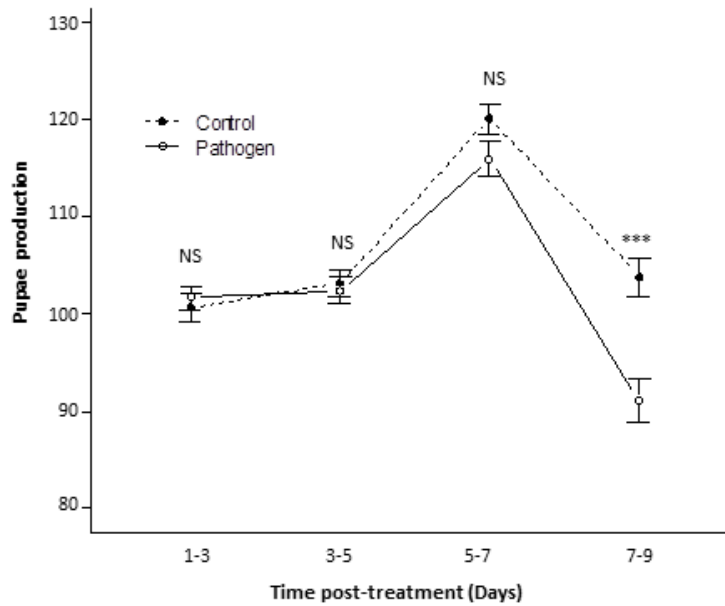


Figure S3: STIs cause fecundity loss for all genotypes. * indicates level of statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Student's t-test was performed for contrast at each time point

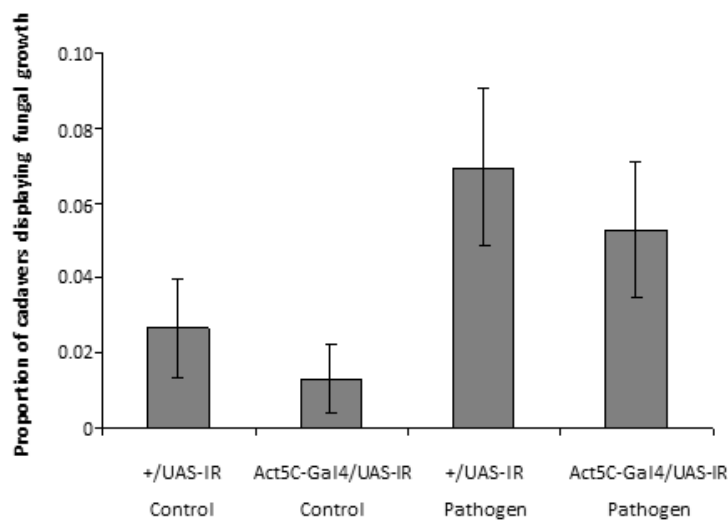


Figure S4: Exposure to fungus-infected male partners increase the likelihood of *Metarhizium*-like fungal growth on cadavers